University of Alberta

THE PHYLOGENY OF THE HEMICHORDATA AND ECOLOGY OF TWO NEW ENTEROPNEUST SPECIES FROM BARKLEY SOUND

by

Christopher B. Cameron

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

Systematics and Evolution

Department of Biological Sciences

Edmonton, Alberta

Fall 2000

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Degree: Doctor of Philosophy

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Abstract

Hemichordates, as represented by the classes Enteropneusta and Pterobranchia, are pivotal to arguments about both deuterostome and chordate origins. The morphological disparity of the two classes suggests two sharply contrasting hypotheses. The earliest protochordate resembled: 1) a burrowing, worm-like, deposit-feeding enteropneust, or 2) a sessile, suspension-feeding, bryozoan-like pterobranch. This thesis presents new evidence that enhances our understanding of deuterostome origins and hemichordate functional biology.

Separate cladistic analyses of morphological and molecular (18S rDNA) characters yielded two parallel conclusions. The Hemichordata are: a) monophyletic, and b) sister group to the Echinodermata. These relationships strong imply the ancestral adult deuterostome: 1) was mobile, not sessile, and 2) possessed a pharynx with gill slits. The placement of the Pterobranchia within the Enteropneusta differed between the analyses, so more data are still needed.

Detailed studies of the form and function of enteropneusts also yielded insights into their biology and evolution. A new species of enteropneust from Barkley Sound, British Columbia, Canada (*Harrimania planktophilus*; Harrimaniidae) was described. Development was direct, but the lecithotrophic larva retained traces of a telotrochal swimming band, suggesting a planktonic ancestry. Adults brooded larvae within their tubes, were active burrowers with an unusually robust body, and possessed many large gill slits with well-developed tongue bars.

Most significantly, *Harrimania planktophilus* was capable of significant subsurface filter feeding. They pumped water rapidly through the mouth (0.5 - 2 mm/s), effectively captured suspended particles down to 0.2 μ m, but showed no evidence of an endostyle or mucous-net capture seen in other chordates. A filter-feeding pharynx therefore seems to have evolved before the divergence of enteropneusts, echinoderms and chordates.

Histological and electrophysiological studies of *Saccoglossus* sp. A revealed: a) dorsal collar and ventral trunk nerve cords composed of small, longitudinal axons, with no evidence of an integrative center, and b) compound action potentials through-conducted from proboscis to trunk. Conduction velocities and ultrastructure suggest the nerve cords are not comparable to the CNS of chordates, but more closely resemble the ectoneural nervous system of echinoderms. Remarkably, histochemical studies revealed GnRH — long considered a chordate neuropeptide — in the nervous system of *Saccoglossus* sp. A and *Ptychodera bahamensis*, where it probably functions as a pheromone.

"All things are one thing and that one thing is all things - plankton, a shimmering phosphorescence on the sea and the spinning planets and an expanding universe, all bound together by the elastic string of time. It is advisable to look from the tide pool to the stars and then back to the tide pool again."

John Steinbeck on Ed Ricketts philosophy. From The Sea of Cortez

Dedicated to Jocelyn and Sandy

Acknowledgements

Most of all I would like to thank my supervisor A.R. Palmer for his unwavering support, both financially and scientifically throughout the past five years of my degree. I would not have been able to accomplish this thesis without his guidance, encouragement and enthusiasm. Rich strives for high scientific standards, has always had faith in my abilities, and most of all has provided me with a tremendous role model. I hope that I can live up to the example that Rich has provided for me.

Thanks are also due to the members of my supervisory committee, Edward Ruppert, William Bates, Andy Spencer (who unfortunately could not attend my defense), and George Pemberton (who stepped forward in Andy's absence) for their advice and assistance. I am especially grateful to Ed Ruppert for sharing his knowledge of the hemichordates and of all invertebrates. Many of the ideas presented in this thesis would not have developed without many long discussions with Ed, both via e-mail and during my visit to Clemson University. I am also grateful for the company and southern hospitality of Jennifer Frick, especially during my visit to their humble home in Balsam Grove, North Carolina.

I would like to express my appreciation to George Mackie for his guidance during the first eight months of my degree, he is an exemplary scientist who gave me confidence in my investigative abilities, and has been with me throughout my thesis work with advice, encouragement and financial support. I am grateful to Nancy Sherwood for providing me with GnRH antibodies, and for her warm invitation to work among those in her lab. The 18S rDNA study presented in this thesis was done in collaboration with Billie Swalla. She is a talented molecular biologist and she loves the animals with which she works. I am grateful to her and the Molecular Biology Research Group and Vanderbilt University, Tennessee, for teaching me "the way of the gene". Billie also provided me with funding to search for *Protoglossus koehleri* from the Aber of Roscoff, France.

Many people have provided me with facilities and support throughout my thesis including the University of Alberta, Department of Biological Sciences, Mary Rice, director

of the Fort Pierce Marine Lab, Florida, Kirby-Smith of the Duke University Marine Lab, North Carolina, the director and staff of the Baruch Marine Field Lab, South Carolina, the director and staff of the Bermuda Biological Station for Research, and the director of the Bermuda Aquarium and Zoo, Wolfgang Sterrer. Most of all I am sincerely grateful to the directors, and staff of the Bamfield Marine Station, and to my friends who live in Bamfield, a truly magical and mystic town. The Cape Beale lighthouse keepers Norby and Cathy Brand (and their dog 'Buck') provided me with living and working space during my visits to collect intertidal *Saccoglossus* and *Harrimania planktophilus*, I would not have been so eager to spend wet winter nights digging in the sand flats without the shelter that they welcomed me into.

Thank you's are extended to the members, past and present in the lab of A.R. Palmer. I had the pleasure of working along side of Timothy Rawlings, Graeme Taylor, Stef Zaklan, Brian Penney, Dave Arsenaut and Jayson Gillespie. I am grateful to all of the SCUBA diver buddies that made it possible for me to work under water during years of shifting rules, regulations, guidelines and policies at BMS. I sincerely hope that underwater research remains a focus and strength of this marine facility.

I would like to single out a few people who I have been especially close to during my graduate career. Mike Swallow has always been ready with critical advice, an open ear and bottomless generosity. He accompanied me with shovel in hand on coastlines from Oregon to the Sea of Cortez. My research on Baffin Island would not have been possible without the friendship of Dave and Dawn Pier. Jayson Hodin manages to occupy a space where freaks are welcome and learing is unavoidable, he has been an exceptional teacher and is a blessed friend. My paleontologist comrads (*Nephtys* warriors) Kevin Brett, Tom Saunders, Murray Gingras, J.P. Zonneveld and Chad Harris for the mind expanding experiences that we shared during many Alberta fossil trips and coastal burrow explorations. The last two years of my thesis work has been difficult, I struggled for a long time before being diagnosed with Addison's disease, and I am sure I was equally difficult to deal with while writing this thesis. I am indebted to Carolyn Bergstrom, and her pets.

Finally, I would like to thank my parents, Jocelyn and Sandy Cameron, for all their love and support, and my immediate family Bruce, Erin, Tyler, and Rylan and Heather, Jeff and Ciaran for always welcoming me "home" when I needed it.

TABLE OF CONTENTS

Chapter Page #

I. **General Introduction** 1

Literature Cited 6

II. **A cladistic study of hemichordate morphology suggests that *Tornaria hubbardi* (Ritter) represents a more appropriate ancestor to the deuterostomes than Garstang's (1928) auricularia** 10

Introduction 11

Materials and Methods 13

Results 14

Discussion 15

- Autapomorphies of major groups 15
  - Enteropneusta 15
  - Pterobranchia 17
  - Echinodermata 18
  - Urochordata 18
  - Cephalochordata 18
  - Craniata (Vertebrata + hagfish) 19
- Synapomorphies of sister groups 19
  - Enteropneusta + Pterobranchia (= Hemichordata) 19
  - Hemichordata + Echinodermata 20
  - Urochordata + Cephalochordata + Craniata (= Chordata) 22
- Deuterostome symplesiomorphies 22
- Modifying Garstang's auricularia hypothesis 25

Literature Cited 37

Appendix II-1 Morphological characters used to construct the deuterostome phylogenetic trees 53

Appendix II-2 Character matrix used in morphological cladistic analysis 59

III. Evolution of the chordate body plan: New insights from a hemichordate molecular phylogeny 60
Introduction 61
Materials and Methods 62
  Animals 62
  Sequences 62
  Alignments and analysis 63
Results 63
  Relationships within the deuterostome taxa 63
  Relationships within the hemichordates 64
Discussion 64
  Deuterostome phylogeny 64
  Colonial pterobranchs likely evolved from solitary enteropneusts 65
  Implications for the evolution of the chordate body plan 67
Literature Cited 74

IV. The anatomy, life habits and later development of a new species of enteropneust, *Harrimania planktophilus* (Hemichordata: Harrimaniidae) from Barkley Sound 76
Introduction 77
Materials and Methods 78
Taxonomic Treatment 79
Harrimania planktophilus new species 79
Description 79
  External 79
  Anatomical and histological characters 80
    Proboscis 80
    Collar 80
    Trunk 81
Diagnosis 81

Development 81

Biology 83

Etymology 83

Holotype 84

Paratypes 84

Evidence for the monophyly of the genus *Harrimania* 84

Phylogenetic relationships 85

Keys to the families and genera of the Enteropneusta, and to the species of the genus

Harrimania 85

Literature Cited 97

V. **Conduction pathways in the nervous system of *Saccoglossus* sp. (Enteropneusta)** 101

Introduction 102

Materials and Methods 103

Results 104

Histology and ultrastructure 104

Behavioural observations 105

Electrophysiology 105

Discussion 106

Literature Cited 113

VI. **Gonadotropin-releasing hormone in mulberry cells of *Saccoglossus* sp. and *Ptychodera bahamensis* (Hemichordata: Enteropneusta)** 115

Introduction 116

Materials and Methods 117

Immunocytochemistry 117

Sections 118

Extraction of peptides 118

Sep – Pak high performance liquid chromatography (HPLC) 118
Purification of GnRH 119
Radioimmunoassay (RIA) 119
Results 120
Mulberry cells in *Saccoglossus*: structure and immunolabelling 120
Mulberry cell distribution in *Saccoglossus* 121
Mulberry cells in other hemichordates 121
Characterization of *Saccoglossus* GnRH 121
Discussion 122
Literature Cited 129

VII. **Particle retention and flow in the pharynx of the enteropneust *Harrimania planktophilus*: The filter feeding pharynx evolved prior to the divergence of hemichordates – echinoderms and the chordates** 133
Introduction 134
Materials and Methods 136
Results 137
Histology and microstructure 137
Observations of flow 138
Particle retention 139
Discussion 140
Literature Cited 150

VIII. **General Conclusions** 154
Cladists, hemichordate phylogeny, and chordate origins 154
Harrimania planktophilus (n. sp.) 156
The filter – feeding pharynx evolved before the divergence of hemichordates and chordates 157
The nervous system of *Saccoglossus* sp. 158

Literature Cited 160

Appendix

I. Brominated compounds secreted by *Saccoglossus* sp. deter fish predation, inhibit aerobic bacterial growth and filter colonization of sympatric sedimentary macrofauna 161

Introduction 162

Materials and Methods 163

  Microbial experiments 163

    Isolation of marine bacteria 163

    Extraction 163

    Preparation of bromophenolated paper disks for bacterial growth assays 163

    Bioassays 164

  Palatability experiment 164

  Colonization experiment 165

Results 165

  Microbial experiment 165

  Palatability experiment 166

  Colonization experiment 166

Discussion 167

Literature Cited 176

LIST OF TABLES

Page #

III-1 A table of the deuterostome (and outgroup) species, GeneBank accession number and taxa that were used in a phylogenetic analysis using the 18S rDNA gene 71

V-1 Lengths and velocities of conduction pathways in the nerve cords of *Saccoglossus* sp. 112

VI-1 Steps in the HPLC purification of GnRH from *Saccoglossus bromophenolosus*. ...124

AI-1 Charcateristics of the aerobic marine bacterium isolated to test for inhibitory growth effects of 2, 4 - dibromophenol and mucus extracted from *Saccoglossus* sp. 170

AI-2 Concentration of 2, 4 - dibromophenol added to paper disks to test growth inhibition of aerobic bacteria plated on agar media 171

AI-3 Shannon index of diversity calculated from the pooled data from summers 1998 and 1999 173

AI-4 Distribution of taxa that colonized control sedimentary containers, hemicontainers adjacent to *Saccoglossus* sp. (and therefore permeated with diffusable halogenated compounds) and containers containing *Saccoglossus* sp. (and therefore susceptible to diffusable halogenated compounds and the mechanical activity of the worms) ...173

LIST OF FIGURES

Page #

II-1 Bootstrap 50% majority-rule consensus tree of the deuterostomes obtained from 79 morphological characters with a heuristic search under the assumptions of parsimony 28

II-2 One of six equally parsimoneous trees of the deuterostomes obtained from morphological characters with a heuristic search, with random stepwise and 200 replications, and TBR branch swapping with MULPARS option on, and steepest descent off 29

II-3 Drawings of a generalized enteropneust 31

II-4 Drawing of a generalized pterobranch based on *Cephalodiscus*. Pterobranchs are clearly the sister group to the enteropneust worms, though they have a U-shaped digestive tract and ciliated tentacles as adaptations to a tube dwelling life and filter – feeding 33

II-5 A) *Tornaria hubbardi* is a living representative of the ancestral deuterostome morphology. *Tornaria hubbardi* has paired eyes, serial paired gill pores and a rudimentary notochord. B) An idealized auricularia larva (modified from Gemmill 1914) demonstrates the homologous characters shared between the auricularia of echinoderms and the tornaria of enteropneusts 35

II-6 Synapomorphies of the deuterostomes are indicated at the root of the tree. Character numbers at the internodes are accompanied by the state changes (in parenthesis). The character numbers correspond with the character numbers in Appendix 1. 36

III-1A Phylogenetic trees illustrating evolutionary relationships among deuterostome taxa as inferred from 18S rDNA sequence analysis. Maximum parsimony using heuristic searches with stepwise random addition of taxa and TBR branch swapping the MULPARS option in effect, and collapsed zero-length branches 68

III-1B Phylogenetic trees illustrating evolutionary relationships among deuterostome taxa as inferred from 18S rDNA sequence analysis. Neighbor - joining trees were constructed using Kimura's two-parameter distances 69

III-2 Cladogram of deuterostome groups for which there is support from molecular and morphological data sets 70

IV-1A Photograph of the adult enterpneust *Harrimania planktophilus* 88

IV-1B Light micrograph of a longitudinal section of *Harrimania planktophilus* 90

IV-2 *Harrimania planktophilus*. Light micrographs of transverse sections 92

IV-3 Schematic drawings of the later development of *Harrimania planktophilus*. A. Blastula. B. Gastrulation. C. Ciliated ball with vestigial telotroch. D. Newly hatched juvenile. E. Juvenile with distinct body regions. F. Juvenile at first gill pore stage. G. Juvenile at second gill pore stage 94

IV-4 Schematic drawing of *Harrimania planktophilus* in situ with cylindrical mass of embryos 96

V-1 Transverse sections through the collar cord of *Saccoglossus* sp.. (A) Paraffin section through the collar cord, showing the nerve fibre layer (nf) beneath the epidermis (ep). (B) Electron micrograph of a section through the fiber layer in a region corresponding to that indicated by the arrowhead in A 110

V-2 Size-frequency histogram of axon diameters measured in the collar cord and ventral trunk cord of *Saccoglossus* sp. 111

V-3 Shock-evolved compound action potentials recorded extracellularly between two points on the dorsal midline of the proboscis (A) and ventral midline of the trunk (B) of *Saccoglossus* sp. 111

V-4 Conduction velocities measured between various points along the dorsal and ventral midlines of *Saccoglossus* sp. (for details of paths see Table 1 112

VI-1 Mulberry cells in *Saccoglossus* shown by immunofluorescence (A,B) and transmission electron microscopy (C,D). A) GnRH-like immunoreactivity in mulberry cells scattered through the posterior collar epidermis, dorsal to the collar nerve cord. B) higher magnification, cells extending from the dorsal collar nerve cord. C) apical portion of a mulberry cell in the mid-collar region, with granules. D) transmission electron micrograph showing granules, nucleus and tapering base of mulberry cell running down into nerve fibre layer 126

VI-2 The distribution of GnRH immunoreactive mulberry cells in *Saccoglossus* sp.. A) dorsal view showing mulberry cells in the general proboscis epithelia with increasing density in the region of the gill pore, collarette, and overlying the dorsal collar cord, B) ventral view of the mulberry cell around the collarette, and with the trunk segment cut mid-ventrally to show the distribution of immunoreactivity in the gill bar epithelia ...127

VI-3 HPLC analysis of immunoreactive GnRH from *S. bromophenolosus* extract. A) irGnRH in eluates from a Sep Pak column with TFA in the mobile phase, B) irGnRH in eluates from a C18 column with TEAF in the mobile phase, C) irGnRH in eluates from a C18 column with TFA in the mobile phase, D) irGnRH in eluates from a phenyl column with TFA in the mobile phase. Solid lines indicate % acetonitrile in the mobile phase 128

VII-1 Scanning electron micrographs (A - D) and light micrographs of sectioned material of *Harrimania planktophilus*. A. The doral branchial pharynx of *H. planktophilus* showing primary and secondary gill bars. B. The gill bars and slits in the dorsal branchial pharynx. C. details of the cilia and mucus on a gill bar. D. The post - pharyngeal organ concentrates food in passage from the pharynx to the gut. E. Light micrograph of longitudinal section through the proboscis, collar and pharyngeal trunk. F. Light micrograph of a sagittal section through the pharynx 145

VII-2 Dye flow into the mouth and out of the gill pores of *Harrimania planktophilus* 147

VII-3 Still photographs captured from video tape of particles moving towards the mouth of *Harrimania planktophilus* 149

AI-1. The mean area of inhibition of aerobic bacterial growth by decreasing concentrations of 2, 4 - dibromophenol, of mucus collected from *Saccoglossus* sp., of paper disks without 2, 4 - dibromophenol or *Saccoglossus* mucus 175

Chapter I

Introduction

This thesis attempts to clarify the phylogeny of the Hemichordata, using cladistic methodology and a combination of new and traditional characters drawn from morphology, molecules and physiology. The Hemichordata is a deuterostome phylum divided into three extant classes (Enteropneusta, Pterobranchia and Planktosphaeroidea) and one extinct class (Graptolithina). The enteropneusts and pterobranchs are so different that many zoologists assign each the status of phylum (Nielsen 1994, Jeffries 1986). Enteropneusts, or acorn worms, typically burrow in sand or mud and ingest large quantities of sediment using a ciliated, muscular proboscis (Barrington 1940, Knight-Jones 1953). They are a common element of the sedimentary coast fauna throughout the world (Horst 1939). In North America many more species have been discovered than are currently described (Ricketts et al. 1985). The Pterobranchs are small colonial animals that inhabit tubes and feed on suspended particles using one or more pairs of tentaculated arms (Lester 1985). Until recently, most of the specimens had been collected by dredging off of Japan, the Atlantic coast of Europe, in the Indopacific, and in Antarctic waters (Horst 1939). Now, however, pterobranchs are being found in shallow water around Bermuda, Fiji and Florida, where they live upon coral reef rubble. The Planktosphaeroidea is represented by transparent spherical larvae – perhaps of some as yet unknown enteropneust - that are about 10 mm in diameter. They are included among the hemichordates because they resemble the enteropneust tomaria (Hyman 1959).

Uncertainty regarding the phylogenetic position of the hemichordates has existed throughout the study of these animals. When the first hemichordate, *Ptychodera flava*, was discovered, Eschscholtz (1825) thought it was an unusual holothuroid. After studying aspects of enteropneust development and noting the presence of an anterior extension of the buccal cavity, the stomochord, which resembles the chordate notochord, Bateson (1884) proposed that the hemichordates be included among the chordates. Sars (1873) discovered the first pterobranch, *Rhabdopleura*, and because of the ciliated feeding tentacles considered it as sister taxon to the lophophorates. It was not until the discovery of the second genus, *Cephalodiscus*, that pterobranchs were united with enteropneusts in the phylum Hemichordata because both taxa had a perforated pharynx and stomochord (Harmer 1887, Hyman 1959).

The notochord and gill slits are traditionally thought of as chordate features, yet both appear to exist in the hemichordates. The dorsal hollow nerve cord, also a chordate feature, may also exist in the enteropneust hemichordates. Consequently, many researchers interested in chordate origins have included hemichordates in their evolutionary hypothesis (St-Hilaire 1822, Patten 1890, Garstang 1928, Berrill 1955, Gutmann 1981, Jefferies 1986, Nübler-Jung and Arendt 1996). Hypotheses about chordate origins and deuterostome evolution have been handicapped because there has been only sporadic interest in the hemichordates since the pioneering work of Spengel (1893, 1901, 1907).

For many years after its discovery the tornaria larva was considered the larva of an asteroid (Hyman 1959). In both larvae, the ciliated bands take a similar course around the mouth, and the digestive system is subdivided into foregut, stomach and intestine. Their early embryology is deuterostomous and cleavage is radial, but perhaps the most convincing evidence for a close relationship between echinoderms and hemichordates is the details of coelomogenesis (Gemmill 1914). In both the coelom generally forms by enterocoely into three successive parts, the anterior protocoel (echinoderm axocoel), the mesocoel (hydrocoel), and the posterior metacoel (somatocoel). The protocoel develops a duct and pore on the left side in the two groups, and forms a heart vesicle (dorsal sac) that is positioned close to an excretory glomerulus (axial gland) (Gemmill 1914, Ruppert and Balser 1986).

This thesis asks the question, what is the relationship among the hemichordates? How do we reconcile the apparent paradox that at one and the same time, they resemble the chordates and the echinoderms? Given the disparity of opinion regarding the phylogenetic relations of the hemichordates, the central purpose of this thesis is to investigate hemichordate relations using modern cladistic arguments. Rather than using one or two morphological characters, for example the stomochord and gill slits, I establish the position of the hemichordates in the deuterostomes by using all of the phylogenetically informative characters that I could acquire. Detailed characters (i.e., stomochord, gill slits, nervous system anatomy) were reduced to smaller, more manageable components. Other, previously unrecognized characters were gathered from the scientific literature, and a few were generated during my own investigations (chapter II).

Of course, a phylogenetic tree is only as good as the data used to construct it, and the tree topology generated from my morphological data is only a hypothesis. However, molecular data are commonly used as an independent data source to test morphological phylogenetic hypothesis. Large amounts of sequence data can be obtained with relative ease and subjected to the newest maximum likelihood techniques, based on models that lend themselves to statistical comparison. In collaboration with Dr. B.J. Swalla (Univ. of Tennessee) we chose the 18S rDNA gene to construct a hemichordate phylogeny because it is commonly used across many taxa, has many known primers, and has been informative for the echinoderms and chordates (chapter III). I collected the pterobranch *Cephalodiscus gracilis* and the enteropneust *Ptychodera bahamensis* from Bermuda. The 18S rDNA sequence of *Saccoglossus cambrensis, S. kowalevskii* and *Balanoglossus carnosus* were obtained from GenBank; and from Barkley Sound, I collected two apparently undescribed species: one of *Saccoglossus*, and another of *Harrimania*.

The most conspicuous characteristics of the new species of *Harrimania planktophilus* is that it had a short proboscis and large gill pores. This peculiar arrangement of proboscis and gill pores led me to propose that this species might use its large pharynx to filter food from sea water. The gill slits of the enteropneust pharynx are generally considered to vent excess water that enters the pharynx with ingested sediment (Barrington 1940, 1965, Knight - Jones 1953, Burdon - Jones 1962) and may function as a gas exchange surface (Ruppert and Barnes 1994). In contrast to the enteropneust pharynx, that of protochordates is a filter feeding surface (ascidians, amphioxus, and appendicularians) (Flood and Deibel 1998), and provides a means of locomotion for salps and doliolids (Bone 1998). The pharynx of lamprey ammocete larvae function much like that of amphioxus (Halstead 1969), and in the vertebrates the pharynx provide a surface for gas exchange (although suspension feeding has been reinvented in some taxa). In higher vertebrates the gill bars have been modified to form the jaws and the hyoid arches (Radinsky 1987). Given the important role that the pharynx has had in the diversification and specialization of the chordates, I embarked on an investigation of the structure and function of the pharynx of *Harrimania planktophilus* (chapter VII).

H. planktophilus is demonstrated to be a suspension feeder, and in chapter IV a complete taxonomic description is provided in addition to observations of its later development. At least 10 enteropneust species are known to occur on the Pacific coast of North America, yet only three, including *H. planktophilus*, have been properly described.

Little is also known about the biology of *Saccoglossus*. From Barkley Sound, *Saccoglossus* sp. was believed to be rare, but it is common subtidally along rocky shores where there is little terrigenous input and substantial amounts of biogenic calcium carbonate sediment. *Saccoglossus* sp. typically lives in a high - energy environment, and consequently burrows rapidly when disturbed. While collecting *Saccoglossus* sp., a diver must approach the bright orange proboscis without touching the ocean floor to prevent *Saccoglossus* sp. from effecting a quick escape into its burrow. This sensitivity suggests that *Saccoglossus* sp. cannot detect predators in the water column, such as fish, but can detect epibenthic predators, such as crabs.

A quick escape response suggests a fast neural conduction pathway. In collaboration with G.O. Mackie (Univ. of Victoria) I recorded conduction velocities from the nervous system (chapter IV) of *Saccoglossus* sp. and examined the dorsal cord of using transmission electron microscopy. We concluded that the nervous system of *Saccoglossus* sp. is equivalent to the ectoneural system of echinoderms. Surprisingly, the prototypical chordate neural peptide gonadotropin releasing hormone (GnRH) was expressed in *Saccoglossus* sp. (chapter V). If *Saccoglossus* sp. avoids epibenthic predation by quickly withdrawing into their burrows, how does it avoid predation by swimming predators?

In collaboration with J. Gillespie I tested the palatability of *Saccoglossus* sp. tissue to crabs and fish. Fish unequivocally reject ingested *Saccoglossus* sp. tissue whereas crabs eat it. These results suggest that the quick escape response in *Saccoglossus* sp. may have evolved to avoid predation by crabs, whereas the unpalatability and aposematic colouring may have evolved as a warning against visual predators. Most species of enteropneusts tested have high concentrations of bromophenols in their tissue (Woodin et al. 1987). *Saccoglossus* sp. smells strongly of bromophenols, and this is the most likely reason that tissue is rejected by fish. In Appendix I present a broad look at the function of bromophenols in *Saccoglossus* sp. Do bromophenols inhibit aerobic bacterial growth? How do they effect colonization by animals occurring sympatrically with *Saccoglossus* sp.? Although bromophenols have so far not provided any information about the macroevolution of enteropneusts, some researchers believe that they may provide a character from which we may characterize *Saccoglossus* clades (King et al.1995). The study presented in the appendix originally began as a side project, but justifies its place in this thesis because bromophenols are undoubtedly one of the most interesting and conspicuous traits of *Saccoglossus* sp. and many other species of enteropneust.

It is hoped that the research presented in this thesis improves our knowledge of hemichordates, and leads to further profitable research on this biologically interesting and evolutionarily pivotal phylum of deuterostomes. As with the dinosaurs, once thought to be unadaptable and sluggish, further research on both groups is revealing a highly adaptable and dynamic group of organisms.

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Chapter II

A Cladistic Study of Hemichordate Morphology Suggests that *Tornaria hubbardi* Better Approximates the Ancestor of the Deuterostomes than does Garstang's (1928) Auricularia

Abstract A cladistic analysis of Hemichordata and higher deuterostomes was conducted using 86 embryological, larval and adult morphological characters, and using the cnidarians, sipunculans, polychaetes and phoronids as outgroup taxa. The pterobranchs (2 genera), and enteropneusts (13 genera) were compared to the echinoderms, urochordates, cephalochordates and vertebrates. Phylogenetic trees were inferred using PAUP 4.1.1 under the assumptions of parsimony using bootstrap method with branch and bound search. Maximum parsimony resulted in 6 equally parsimonious trees that differed in the placement of the enteropneust *Stereobalanus* and the morphologically complex ptychoderid worms in relation to other enteropneust taxa. All trees examined placed the enteropneusts as the sister group to the pterobranchs, and the monophyletic hemichordates as the sister group to the Echinodermata. Hemichordate monophyly is based in part on the presence of a heart/ kidney coelomic complex and paired mesocoelic pores. The hemichordates are the sister group to echinoderms, with which they share a dipleurula larva and a tricoelomate body organization. The Chordata, including the Urochordata (tunicates), Cephalochordata (*Branchiostoma*) and Vertebrata (+ hagfish) were found to be monophyletic based in part on their shared neurulated dorsal cord, endostyle, dorsal post-anal tail and metameric muscles. Deuterostome symplesiomorphies include radial cleavage, deuterostomous development of the gut, enterocoelic formation of the coelomic cavities, a closed Harveyian haemal system, and gill pores. Ancestral character reconstruction using MacClade suggests that the common ancestor, or zootype, of the Deuterostomia was tornaria - like animal with an enteropneust - like morphology in its life history. The echinoderms evolved from the tornaria - like morphology and the chordates from the acorn worm - like morphology. Chordate metamerism evolved as a modification of branchiometry only after the divergence of the chordates with the common deuterostome ancestor. The foundation to evolve a main nerve cord (homologous with the nerve plexus that innervates the gill complex in enteropneusts), and notochord (modified from the stomochord for a change in function) exists in the Enteropneusta.

'Facies non omnibus una, nec diversa tamen, qualem decet esse sororum.'
Their faces were not all alike, nor yet unalike, but such as those of sisters ought to be.
Ovid

Introduction

The enteropneust worms and the lophophorate-like pterobranchs hold a key position in hypotheses about deuterostome evolution, yet their systematic position among deuterostomes is not well established. When the first hemichordate, the enteropneust worm *Ptychodera flava*, was described by Eschscholtz (1825) it was thought to be a holothurian. Members of the class Enteropneusta (Gr. "intestine-lung"), with their numerous gill slits were long regarded as chordates (Bateson 1886). The pterobranch *Rhabdopleura* (Allman 1869; Sars 1873) was originally united with the Bryozoa in the class Pterobranchia (Gr. "wing-gill") by Lankester (1884) based on similarity of form and function of the feeding tentacles. M'Intosh (1887) recognized the relationship of the pterobranch *Cephalodiscus* with its single pair of gill pores to the enteropneust *Balanoglossus* and, together with Fowler (1893), suggested the inclusion of Pterobranchia (including *Rhabdopleura* and *Cephalodiscus*) into the Hemichordata. This idea gained further support from Horst (1939), Dawydoff (1948) and Hyman (1959) in their respective treatises. More recently, Eernisse et al. (1992) and Nielsen (1996) conducted cladistic analyses of morphological character matrices for the Metazoa that suggest, as Bateson (1886) believed, that the enteropneusts are the sister group to chordates based on the shared presence of paired gill slits, and that the pterobranchs are basal to all deuterostomes, in other words that the phylum Hemichordata is polyphyletic. Given the wide range of opinions regarding deuterostome phylogeny (see Gee 1996 for a review of hypotheses of chordate origins) I embarked on a detailed comparative study of deuterostome morphology with particular emphasis on the enteropneust and pterobranch genera.

Not included in this study are the monotypic class Planctosphaeroidea (Gr. "plankton-sphere") and the fossil class Graptolithina. *Planctosphaera pelagica* (Spengel 1932) is a large planktonic larva with elaborate feeding bands, and a locomotory telotroch (Horst 1939; Hadfield and Young 1983; Hart 1994), and likely represents an example of a tornaria that has become highly modified for extended planktonic life. Like Planctosphaeroidea, the adults of most tornaria larvae that are described from the plankton are unknown. *Planctosphaera* and any tornaria that cannot be assigned with certainty to an adult genus are not included in this study. The class Graptolithina is an extensive assembly of fossils that radiated throughout the Ordovician before disappearing from the fossil record in the middle Pennsylvanian (Boardman et al. 1987). Graptolites have been considered as cousins to hydroids and bryozoans but now most authors agree that they are related to pterobranchs (Meichin and DeMont 1995; Urbaneck et al. 1992; Dilly 1976). The soft bodies of graptolites are not preserved in the fossil record. They are considered sister group to pterobranchs (especially *Rhabdopleura*) on account of the ring-on-ring construction of the tube in which the ends of the rings meet along a more-or-less zig-zig suture (Dilly 1976), the presence of a stolon system (lost in the more derived graptolite groups), and the apparent similarities of the early post-settlement growth phases of *Rhabdopleura* with the ontogeny of the graptolite siculum. The fossil record for *Rhabdopleura* is not rich although it does extend back to the Cambrian (Urbanek et al. 1992).

The poor fossil record for enteropneusts consists of relatively recent fossils from the Pennsylvanian (Bardack 1985) the Triassic (Twitchett 1996) and the Sinemurian, which resemble the modern day enteropneust *Balanoglossus* (Arduini et al. 1981). The oldest fossil chordates are of Cambrian age. The earliest is the supposed cephalochordate *Yunnanozoon lividum* from the Early Cambrian (525 Ma) of China (Dzik 1995; Chen et al. 1995). *Pikaia* (Conway Morris and H.B. Wittington 1979) from the Middle Cambrian Burgess Shale fauna is another possible cephalochordate. The calcichordates are uncertainly related to other Chordata (Nielsen 1994; Jefferies 1996). The fossil record and estimates based on molecular clock studies (Wray et al. 1996; Bromham et al. 1998) provide conservative estimates for deuterostome radiation predating 525 mybp. The deuterostomes are a morphologically disparate group that are rooted deeply in Precambrian history, and although the fossil record for the echinoderms and vertebrates is good, that for the pterobranchs, enteropneusts and tunicates is depauperate.

The relationship of the hemichordates to other deuterostome taxa remains an issue of spirited debate. Hemichordates have been considered as sister group to the echinoderms (Horst 1939; Dawydoff 1948; Berrill 1955; Hyman 1959; Jefferies 1986), the cephalochordates (Gutmann 1981), and the chordates (Siewing 1972). Studies employing 18S rDNA sequence data to address the question of deuterostome phylogeny find support for a hemichordate-echinoderm clade and weak support for a chordate clade (Turbeville et al. 1994; Wada and Satoh 1994; Halanych 1995). The old idea that the chordates are inverted dorso-ventrally (Hilaire 1822; see Nübler-Jung and Arendt 1994 for a review) with respect to protostomes is reemerging with alternate interpretations of morphological features (Kirsteuer 1969; Malakhov 1977; Bergström 1997) and new molecular insights into body plan development (Arendt and Nübler-Jung 1997).

Given the wide range of opinions regarding hemichordate phylogeny, I embarked on a project to create a robust phylogeny for deuterostomes, with particular emphasis on the enteropneusts and pterobranchs, using embryological, larval and adult morphological characters.

Materials and Methods

Specimens of adult *Saccoglossus* sp. (British Columbia), *Harrimania planktophilus* (British Columbia), *Ptychodera bahamensis* (Bermuda), *Balanoglossus aurantiacus* (North Carolina), *Glossobalanus berkeleyi* (Washington), *Cephalodiscus gracilis* (Bermuda), *Rhabdopleura normanii* (Bermuda), *Branchiostoma floridae* (Florida) and members of other deuterostome taxa were collected, dissected, and drawn with the aid of camera lucida using a variety of zoom stereo microscopes and usually an Olympus BH-2 compound microscope with Nomarski differential interference contrast. At least one representative member from each pterobranch and enteropneust family (representing 5 families and not including the enteropneust family Saxipendidae) were embedded in either EPON wax or Spurrs plastic (JBS supplies, J.B. EM Services Inc., Dorval, Quebec) for sectioning and observed with a light microscope.

The data set was constructed from the morphological study of the taxa listed above, and by a critical review of the deuterostome literature; particularly important were the writings of Horst (1939), Dawydoff (1942), Hyman (1959), and Benito and Pardos (1997). Eighty-six parsimony informative characters (Appendix 1) were chosen. A total of 23 taxa was examined including two pterobranch families, 13 enteropneust genera (from 4 families), the echinoderms, urochordates, cephalochordates, vertebrates and the cnidarians, polychaetes, sipunculids and phoronids as outgroups. Phylogenetic analysis was performed using PAUP 4.1.1 (Swofford 1999). Most characters were treated as binary (usually absent or present) and one character was coded as a multistate character (75; haemal system) and unordered. For characters scored as absent or present: 0 = absent, 1 = present. Other character states used were 'P' (0 and 1, or polymorphic) and '?' (unknown). All characters were treated with equal weight.

Trees were constructed under the assumptions of parsimony using bootstrap method with branch and bound search (see Fig. 1). The sister group of the very diverse Deuterostomia is presently not known, so I chose 4 phyla, including Cnidaria, Polychaeta, Sipuncula, and Phoronida, and compared each on their own as an outgroup taxon and then combined as polyphyletic outgoup taxa. Six equally parsimonious trees were obtained with a heuristic search, with random stepwise addition and 200 replications, and TBR branch swapping with MULPARS option on, and steepest descent off (Swofford 1999) (see Fig. 2). Statistics reported here from the bootstrap branch and bound search and from the heuristic search include tree length (TL), consistency index (CI) and retention index (RI).

The ancestral reconstructions reported for this analysis include the three character optimization algorithms available in MacClade: ACCTRAN, DELTRAN, and MIN F (Maddison and Maddison 1992) and from the apomorphy list in the tree statistics available in PAUP (Swafford 1999). Character transformations were reconstructed using MacClade 3.01 (Maddison and Maddison 1992).

Results

This phylogenetic analysis of the deuterostome clade used 86 embryological, larval and adult morphological characters (Appendix 1) because they were phylogenetically informative among the deuterostome taxa, and particularly among the hemichordates. Non-deuterostome taxa (Cnidaria, polychaete, Sipuncula and Phoronida) were employed as outgroups. A bootstrap method with a branch and bound search supported the enteropneust clade 99 times out of 100, and the pterobranch clade 91 times out of 100 (Fig. 1). Bootstrap values for the monophyly of the hemichordates, and the echinoderms + hemichordate sister group relationship, respectively, are 82 and 93 (Fig. 1). These nodes were supported on all six equally parsimonious tree topologies observed using a heuristic search with random stepwise addition and equal weighting (Fig. 2) (the strict consensus tree had the identical tree topology to the bootstrap branch and bound tree). The six equally parsimonious trees differed only in the arrangement of the enteropneust genera. In 3 of 6 trees the family Ptychoderidae (P) (+ Xenopleura) was basal to the remaining enteropneust families and the Spengelidae (Sp) (+ the monotypic Saxipendidae (Sx)) formed a monophyletic clade (Fig. 2). Among the enteropneusts, the morphologically complex and primarily indirect developing ptychoderids may be an early offshoot from the worm lineage and the morphologically simple Harrimaniidae (H), a family composed primarily of direct developers, and including *Protoglossus* may be derived more recently within the Enteropneusta (Fig. 2). Bootstrap values obtained for the branches leading to *Xenopleura* plus the ptychoderid worms and to *Saxipendium* plus the spengelids were low (and not included here) and these relationships are admittedly dubious. Among the Harrimaniidae, relations among genera (*Protoglossus*, *Saccoglossus*, *Harrimania* and *Stereobalanus*) were unresolved in the 6 most parsimonious trees (Figs. 1 and 2). Among the chordates, the Urochordatata formed a weak sister group to the cephalochordates (Fig. 2) and together with vertebrates (+ hagfish) formed the well suported and monophyletic Chordata (Fig. 1). The chordates were the sister group to the echinoderms + hemichordates, together comprising the deuterostomes with bootstrap value 87 (Fig. 1). Tree statistics for the bootstrap analysis with branch and bound search were TL - 124, CI - 0.701, RI - 0.791 (Fig. 1) and the six minimum length heuristic trees were TL - 132, CI - 0.674 and RI - 0.829 (Fig. 2).

Accelerated transformation (Acctran), and delayed transformation (Deltran) were tried in a heuristic search and made no difference in tree topology. Reconstruction of ancestral character states indicated that the ancester to the chordates (hemichordates + echinoderms) had eggs with a fertilization membrane that were fertilized externally. Cleavage was radial and coelom formation was primarily by enterocoely resulting in three coelomic cavities. The most anterior coelom was unpaired and encased a heart with a filtration glomerulus and a canal pressurized with a pericardial coelom and lined by podocytes that directed excretory metabolites to an outer pore. A notochord-like structure with a sheath containing extracellular spaces appeared at some stage during ontogeny. The mesocoel and metacoel were paired. The nervous system was a subepidermal plexus with little regionation beyond an apical sense organ (Fig. 5a).

Discussion

One of the most vexing questions in metazoan phylogenetics is the relationship among the deuterostome taxa, a disparate group of animals within which the chordates evolved. The objective of this study was to provide a detailed morphological review of the fifteen hemichordate genera (from the classes Pterobranchia and Enteropneusta) to determine relationships within the phylum (and indeed whether the Hemichordata is monophyletic) and to develop an hypothesis of the relationship among the echinoderms, enteropneusts, pterobranchs, urochordates (tunicates), cephalochordates (amphioxus) and the vertebrates + hagfish. The autapomorphies of the major groups, synapomorphies of sister taxa and finally the symplesiomorphies of the Deuterostomia are discussed. Ancestral character reconstructions using MacClade (Maddison and Maddison 1992) suggested a detailed hypothetical zootype from which the deuterostome taxa radiated, and fortuitously this ancestral morphology may exist virtually unchanged to the present day in *Tornaria hubbardi* (Ritter and Davis 1904) and the tornaria of *Balanoglossus kowalevskii* (Agassiz 1873; Morgan 1891) (Fig. 5a).

Autapomorphies of major groups

Enteropneusta Enteropneust autapomorphies include the pre-oral ciliary organ (Spengel 1893; Brambell and Cole 1939; Knight-Jones 1953; Welsch and Welsch 1978), the hepatic / branchial pharynx, a Y-shaped nuchal skeleton, perihaemal coeloms correlated with a collar nerve cord, mesocoel ducts which open into the first pair of gill pores, a larval locomotory telotroch and an apical plate retractor muscle (Morgan 1891). Though it is generally believed that the small worms from the family Harrimaniidae is basal among the enteropneusts, and represent an ancestral morphology (Hyman 1959), the opposite is suggested here. The family Ptychoderidae, the morphologically most complex of the enteropneusts with gonadal wings, an intricate gill skeletal complex, hepatic sacs (Fig. 3) and typically developing via a tornaria larva (Fig. 5a) is an early offshoot of the worm lineage. Three of six most parsimonious trees obtained with heuristic search revealed the ptychoderid worms as sister group to the remaining enteropneusts. The remaining three trees do not resolve relationships among the hemichordate families, although the families Spengelidae (Sp) and Ptychoderidae (Pt) are resolved into monophyletic clades (Figs. 1 and 2). Spengelids likely represent an intermediate node sharing characteristics of both the families Ptychoderidae and the Harrimaniidae (H) (Fig. 2).

A heuristic search of the hemichordate clade revealed ptychoderids + *Xenopleura* as the sister group to the remaining enteropneusts (Fig. 2) in 3 of 6 trees. They have a nuchal skeleton (Fig. 3) with short horns and a trunk that is regionated into a pharyngeal region with associated gonadal wings (*Balanoglossus* and *Ptychodera*) or ridges (*Glossobalanus*) with lateral septa (Appendix 1, character 50). They have a short esophageal region followed by numerous dorsal hepatic sacculations (Fig. 3). Peribuccal diverticula, anterior projecting extensions of the trunk coelom, invade the collar coelom forming a band of circular muscles around the buccal region. The perihaemal diverticula, also anterior projections of the trunk coelom, extend parallel to the dorsal blood vessel where they develop strong longitudinal muscles (Willey 1899) and most likely function to pressurize blood for flow and ultrafiltration (Balser and Ruppert 1990) (Fig. 3). The gill region of ptychoderids has peribranchial ridges separating the dorsal, or branchial chamber from the ventral or digestive tube (Hyman 1959). The ptychoderids have synapticles in the gill skeleton that join the tongue bars to the primary bars. The post branchial region has paired dorsal ciliary grooves and in some members a ventral cord of turgid vacuolated cells referred to as a pygochord (Uribe and Larrain 1992) apparently also present in *Saxipendium* (Woodwick and Sensenbaugh 1985) and considered by some to be a possible homologue of the chordate notochord (Bergström 1997). Cross sections of *Ptychodera flava* and *Glossobalanus berkeleyi* did not reveal anything resembling a pygochord, and (as corresponded to me by E.E. Ruppert) the initial descriptions of the organ may be an artifact created by the collapse of vessel walls around hemocytes during fixation. The dorsal branchial pores form a ridge that is flanked on either side by genital wings. *Glossobalanus* has gonadal ridges rather than wings and hepatic sacs that are arranged in two dorsal longitudinal rows. Indirect development is assumed to be plesiomorphic for the enteropneusts because ptychoderids primarily develop via a long-lived tornaria larvae (Fig. 5a).

The ‘rock pendulum worm’ *Saxipendium coronatum,* lives atop rocky outcrops adjacent to Pacific deep sea hydrothermal vents (Woodwick and Sensenbaugh 1985). It lacks synapticles and apparently lacks a neuropore. The nuchal skeleton is long, similar to what is found in the family Spengelidae, but it is recurved (Woodwick and Sensenbaugh 1985). It is unknown *S. coronatum* has peribuccal or perihaemal diverticula, confounding efforts to place it phylogenetically and questioning the validity of the monotypic family Saxipendidae. Here it is weakly supported as sister group to the spengelid worms (Fig. 2).

The Spengelidae is an intermediate family of enteropneusts, sharing a combination of ptychoderid and harrimaniid features. Spenglidae has a stomochord that flattens and extends anteriorly to the tip of the protocoel and a nuchal skeleton with horns that extends far into the collar (Hyman 1959). Spengelids lack gonadal wings with lateral trunk septa, and with the exception of *Schizocardium* they lack hepatic sacculations. The two most familiar genera *Spengelia* and *Schizocardium* have synapticles in the gill skeleton and coelomic peribuccal spaces. *Glandiceps* is without either. *Willeyia* has peribuccal spaces but lacks synapticles (Hyman 1959).

Protoglossus koehleri (Burdon-Jones 1956; Vogel 1987) belongs to the family Harrimaniidae, and has traditionally been considered the most primitive of all enteropneusts. *P. koehleri* is characterized as lacking anteriorly projecting perihaemal and peribuccal diverticula of the trunk coelom into the collar coelom (Burdon-Jones 1956). The skeletal apparatus of the gill slits lack tongue bars, and therefore synapticles are also not found. Other members of the Harrimaniidae can also be recognized by the morphological structures that they do not possess. *Saccoglossus* is perhaps the most common of all hemichordates found intertidally and typifies the harrimaniid morphology without hepatic sacculations or genital wings. Peribuccal and perihaemal diverticula and lateral trunk septa are sometimes present. Synapticles are also absent. *Harrimania* and *Stereobalanus* have paired proboscis pores associated with a dorsal proboscis coelom septa, and gill pores fused to a common dorsal slit (Ritter 1900, Reinhard 1942). *Xenopleura*, known from a single described animal may be viviparous, and has a stomochord that extends from the proboscis coelom through the collar where it bifurcates paralleling either side of the dorsal trunk nerve cord (Gilchrist 1925). The taxonomic descriptions of the viviparous *Xenopleura* ("strange side") (Gilchrist 1925) are incomplete and consequently efforts to place them phylogenetically are hampered. Development is direct in harrimaniids; like the pterobranchs they have large yolky eggs and rapid development.

Pterobranchia Pterobranch autapomorphies include their collagenous/ proteinaceous tubes (although they are considered to be homologous with those of .graptolites) (Melchin and DeMont 1995; Rigby and Dilly 1993; Dilly 1976), which they secrete using special cells of the locomotory cephalic shield (protosome), a pigmented band of ciliated cells on the ventral cephalic shield (Horst 1939) of unknown function, a non-migratory mesenchymal, pulsatile vesicle (= pericardium) (Lester 1988b), a U – shaped gut, and mesocoelic arms and tentacles. The tentaculated arms, which contain coelomic extensions of the paired mesocoels, are used in suspension feeding. Although they are sometimes referred to collectively as a lophophore, the feeding structure is convergent to that of the protostome 'lophophorates' (Halanych 1996). Pterobranchs are unique in producing asexual zooids that are linked by a common stolon (*Rhabdopleura*) or germinal disk (*Cephalodiscus*) (Lester 1985) and any one colony may be dioecious or monoecious (Hyman 1959). *Cephalodiscus* females produce two eggs, whereas *Rhabdopleura* have only one (Horst 1939). Development is via a lecithotrophic larva (Gilchrist 1917; Dilly 1973; Lester 1988a) that secretes a collagenous dome-shaped prosiculum under which it metamorphoses and later emerges as a juvenile (Dilly 1985, Lester 1988a). *Cephalodiscus* has a pair of gill pores, whereas *Rhabdopleura* has only a pair of depressions in the endoderm of the pharynx (Horst 1939) suggesting that the gill pores have been lost.

Echinodermata Echinoderm autapomorphies include pentaradial symmetry in most adults, mutable connective tissue, a closed Galenic haemal system, a water vascular system derived from the left mesocoel (hydrocoel) and to a certain extent the protocoel (axocoel), a mesodermal, subepidermal system of calcareous ossicles or plates, the stone canal is unique to the echinoderms (Crawford and Chia 1978). Necrotic metamorphosis of the larval body is widespread among the echinoderms, but is absent in crinoid ontogeny (Clark and Clark 1967) and therefore is not considered here.

Urochordata Urochordates are a monophyletic clade and are unique among the deuterostomes in that they are mainly hermaphroditic (Swalla et al. 2000). During early development urochordates develop a single pair of coelomic cavities that secondarily fuse on the mid-ventral line to form the pericardium, otherwise they have no coelomic body cavities (Berrill 1950). Instead, they have a well developed connective tissue (including the tunic) in which mesenchymal cells enter, move, and differentiate. The larval body is lecithotrophic with a trunk and a locomotory tail (except salps and pyrosomids, which are without larvae) (Jeffery and Swalla 1997). Some of the molgulid ascidians have direct development (Hadfield et al. 1995). They have a perforate pharynx that usually takes up most of the body volume, and a nervous system in the form of a ganglion between branchial and atrial apertures from which neurites radiate (Mackie 1995). Their body is enclosed in a secreted tunic, test or house composed of cellulose and protein, and in which are found migratory cells and extracorporeal blood vessels. Urochordates have periodic heartbeat reversals which result in a change of the direction of flow of blood. The house uses excitable epithelia to transmit information (Mackie 1995).

Cephalochordata Cephalochordates have a laterally compressed body that is lancelet shaped with no appreciable head, or paired sensory organs. Cephalochordates have a ciliated wheel organ before the opening to the mouth (Ruppert 1997b). The notochord extends the entire length of the body, extending to the anterior - most tip (Young 1962, Ruppert 1997a). The lancelet neuraxis is unique in having autofluorescent cells at the anterior end (Fritzsch 1996), although the anterior cord is at least partially homologous to the vertebrate brain (see Lacalli 1996). The bilateral myomeres are out–of–register (rather than in–register, as in vertebrates) mononucleate cells. A slender tail arises from each cell and extends to the nerve cord, at which it recieves motor innervation (Holland 1996). The muscles, nervous system and excretory nephridia are serially arranged. Hatschek's nephridium, is the anterior-most nephridia which opens into the pharynx inside the dorsal lip, the remaining nephridia open into the gill cleft suggesting that the mouth is a serial homologue of the left gill slits (Ruppert 1996). The pharynx, gill slits and gut of amphioxus are enclosed by a ventral atrium, part of which is associated with a pterygeal muscle (Northcutt 1996, Ruppert 1997b). The coelomic cavities of cephalochodates form fin rays in series along the dorsal midline. The conspicuous asymmetries (Ruppert 1997b) observed during ontogeny of amphioxus may be a result of an asymmetric ancestry (Jefferies 1996), but a more likely function is as a means to achieve planktotrophy associated with a small size (Stokes and Holland 1995; Gilmore 1996; Presley et al. 1996).

Craniata (Vertebrata + hagfish) Synapomorphies of the Craniata include the presence of a cartilaginous head skeleton, which surrounds a relatively large brain, plus a unique set of motor and sensory nerves, embryonic neural crest, neurogenic placodes or paraxial mesoderm (Northcutt 1996). The craniates possess a two-chambered heart (Janvier 1981). Craniates form organs by migration and differentiation of mesenchyme cells, whereas organogenesis in hemichordates, cephalochordates and echinoderms is predominantly achieved by extension and differentiation of coelomic diverticula (Ruppert 1997). The traditional taxon Cyclostomata (living lampreys and hagfishes) appears to be a monophyletic assemblage (Mallatt and Sulliven 1998) whereas Agnatha (jawless fishes) and Ostracodermi (fossil jawless fishes) may be non-monophyletic assemblages (Janvier 1996).

Synapomorphies of sister groups

Enteropneusta + Pterobranchia (= Hemichordata) Hemichordate synapomorphies include a muscular-secretory-locomotory pre-oral proboscis that encompasses a heart / kidney coelomic complex, including a stomochord (Schepotieff 1907, Dilly et al. 1986 (for *Cephalodiscus*), Wilke 1972 (*Glossobalanus*), Balser and Ruppert 1990 (*Saccoglossus*)), paired valved mesocoel ducts and pores (Schepotieff 1907), and a ventral post anal extension of the metacoels (Schepotieff 1909; Horst 1939; Hyman 1959) (Figs. 3 and 4). The bootstrap method with branch-and-bound search supports the enteropneust plus pterobranch clade 82 times out of 100 (Fig. 1), supporting conventional views of hemichordate monophyly (Horst 1939, Dawydoff 1948, Hyman 1959). Recent cladistic morphological studies of the Metazoa argue that the enteropneusts are sister group to the chordates, and the pterobranchs are sister group to the echinoderms (Eernisse 1992, Nielsen 1996), however these studies focus on all Metazoa and consequently employ a limited data set to construct the deuterostome clade.

Perhaps the most apparent synapomorphy of the hemichordates is the proboscis (Fig. 3). The enteropneust proboscis is mainly a locomotory, and muco-ciliary deposit feeding organ whereas its equivalent in the pterobranchs, the cephalic shield, is locomotory and secretes the flexible tubes of the coenecium (Lester 1988a). Understanding the biochemical composition of the pterobranch coenecium and the mucus which lines the burrows of the enteropneusts may provide further evidence for homology of the protocoel organs. The hemichordate protocoel encompasses the heart/ kidney coelomic complex, including the stomochord, the contractile pericardium, blood sinus and filtration glomerulus, and a duct and pore (a pair in *Stereobalanus* and all pterobranchs). Blood flows anteriorly in the dorsal vessel of hemichordates and enters the heart sinus. The heart sinus is sandwiched between the contractile pericardium above and the rigid stomochord below (Fig. 3). Contraction of the pericardium pressurizes the blood in the heart sinus promoting circulation into vessels of the or into the glomerulus. The glomerulus is composed of a vessel (pterobranchs) or vessels (enteropneusts) that are overlain by mesodermal podocytes, regionally specialized cells of the protocoelic lining. Some of the pressurized blood is ultrafiltured across the glomerulus and enters the protocoel, as primary urine, before being modified and discharged via the protocoel duct and pore as final urine (Balser and Ruppert 1990) (Fig. 3). Ultrafiltration podocytes are found in the protocoel duct (Ruppert and Balser 1986), and associated with the gill structures (Pardos and Benito 1997) of enteropneusts, and may also be associated with the mesocoel pores and ducts.

Paired mesocoel (collar) ducts and pores indicate a close relationship of the enteropneusts with the pterobranchs. The mesocoel ducts and pores provide a ciliated passage to the outside from the paired mesocoels and may be associated with ultrafiltration sites but it remains to be demonstrated if they have an excretory function. *Rhabdopleura* is unique among the hemichordates in that it lacks a pair of gill clefts, but have paired blind grooves in the pharynx (Hyman 1959) suggesting that the clefts have been secondarily lost. *Cephalodiscus* has a single pair of gill clefts in the anterior trunk (Fig. 4), similar to what is seen in young enteropneust worms. The postanal tail, containing a ventral extension of the posterior metacoels in enteropneust juveniles (Horst 1939, Burdon-Jones 1952), is believed to homologous to the pterobranch stolon (*Rhabdopleura*) and stalk (*Cephalodiscus*) (Hyman 1959, Burdon-Jones 1957, Hadfield 1977, Lester 1985) and functions in locomotion and adhesion.

Hemichordata + Echinodermata For many years after its discovery the enteropneust tornaria was considered the larva of an echinoderm, in particular an auricularia or bipinnaria of an asteroid. The large gelatinous larvae share a preoral larval feeding band that creates an upstream feeding current using monociliated cells (Morgan 1891; Strathmann and Bonar 1976) and a perioral ciliated band that manipulates and conveys food into the esophagus. Though there are many gaps in our knowledge of coelomogenesis in the hemichordates, and what is known is sometimes interpreted from a limited set of ontogenetic stages, the organization of the coelomic sacs nevertheless provides a convincing comparative test of their sister group status. The coelomic sacs in hemichordates and echinoderms are organized anterior to posterior as an unpaired protocoel (echinoderm axocoel), and paired mesocoels (echinoderm hydrocoels) and metacoels (echinoderm somatocoels) (Figs. 3, 4 and 5a,b). Development of the coeloms is primarily enterocoelous, although deviation from the ancestral norm is seen in both phyla (Hadfield 1975). Within the genus *Saccoglossus* coelomic sacs may form via enterocoely, schizocoely or epiboly (see Hadfield 1975 for review). Gemmill (1914) provides a thorough comparative study of coelomogenesis in the asteroid *Asterias* and in enteropneusts, from which much of the following discussion is paraphrased. For an extensive comparison of auricularia to tornaria see Morgan (1891), Gemmill (1914) and Gislen (1930).

The hemichordate protocoel is here considered homologous to the aboral part of the echinoderm axial sinus including the ampullary region, or funnel of the axial sinus (Gemmill 1914). Whereas the right protocoelic cavity is small or absent, the left coelom is dominant and extends a ciliated duct to the exterior via a left dorsal lateral pore. The extension of a coelomic canal to meet a surface hydropore on the left side of the tornaria and all planktotrophic echinoderm larvae possess functional nephridia (mesothelial monociliated podocytes and myocytes) probably involved in extracellular volume regulation (Ruppert and Balser 1986). The coelomic canal is lined by transportive epithelial cells and remains open to the exterior. Primitively the anterior coelomocanal and pore may have been paired (Goodrich 1917; Ruppert and Balser 1990; however see Gemmill 1914 who believed that it was unpaired). The protocoel is pressurized by a contractile pericardial sac (echinoderm dorsal sac) composed of striated muscle that is generally thought to be derived from the protocoel, but may arise from mesenchyme. The larval pore canal-hydropore complex is retained in adult echinoderms as an axial (hemichordate heart-kidney) complex, although there is no homologue of the stomochord in echinoderms. This complex is composed of a glomerulus that is formed from the axial (protocoelic) coelom and axial blood vessels, a heart sinus and pericardium (echinoderm head process and axial organ) at least one coelomic duct and pore which opens to the exterior (echinoderms madreporic pore, or pores).

The left collar coelom of hemichordates is equivalent to the deeper part of the axial sinus, hydrocoel, and the circular sinus (hyponeural ring sinus) which surrounds the mouth of echinoderms (Gemmill 1914). There are no homologues to the right collar coelom of enteropneusts except in some asteroids where a double-hydrocoel is observed.

The right and left trunk coeloms (metacoels) of hemichordates are homologous to the epigastric and hypogastric coeloms (together the perivisceral coelom) of asteroids respectively. The coelomic diverticula of the metacoels in hemichordates (perihaemal coeloms) are homologous to the echinoderm perihaemal coelom (Gislen 1930). The metacoelomic extensions around the collar pharynx (peripharyngeal coeloms) of enteropneusts are homologous to the peripharyngeal coelom of the echinoderms.

The relationship of the tricoelomate condition seen in the echinoderm and hemichordate phyla to the segmental coelomic condition of cephalochordates and vertebrates makes it particularly difficult to establish which (if any) segments in the chordate body plan may be homologues to the protocoels, mesocoels, and metacoels of hemichordates and echinoderms. In other words, if the ancestor to the chordates had three coelomic segments, where were additional segments added to achieve the metameric chordate body plan? Answers to this fundamental question may be unraveled with comparative gene expression data, in particular the expression of the Hox gene complex (Holland 1996), and detailed microanatomical analysis (Lacalli 1996).

Urochordata + Cephalochordata + Craniata (= Chordata) The Phylum Chordata includes the tunicates (Urochordata), lancelets (Cephalochordata), craniates (hagfish and vertebrates), and possibly some odd extinct groups (Romer and Parsons 1986). Chordate monophyly is strongly supported (Fig. 1) with a bootstrap value of 100. Chordate synapomorphies include a notochord, dorsal hollow nerve cord (with an anterior to posterior flow in the neurocoel), dorsal post anal tail, an unpaired pineal eye, visceral clefts, and an endostyle that binds iodine. There is strong morphological, especially embryological, evidence for monophyly of the Urochordata, Cephalochordata and Craniata (Schaeffer 1987).

Albeit a poorly supported clade (Fig. 1) the urochordates and cephalochordates are considered sister group here because of their shared metameric organization (lost, or suppressed in urochordates) and they are united by a secondary multiplication (i.e., exceeding number of body segments) of gill slits. They have no appreciable head, jaws, appendages and paired sensory organs. They share a large perforated pharynx with a branchial siphon (oral hood), atrial cavity and single atriopore. They are mucociliary suspension feeders that secrete a mucus net with an endostyle to capture food suspended in the water column. Evidence that urochordates are chordates comes clearly from their pharyngeal slits and endostyle which persists in larval lampreys and is homologous with the thyroid gland of vertebrates (Leach 1939). Tunicates were united with the chordates with the discovery of their larval tadpole (Kowalevsky 1866), which may approximate the ancestral chordate morphology (Jeffery and Swalla 1997).

Deuterostome Symplesiomorphies

Except for the craniates, deuterostomes are entirely marine and most are microphagous. In a bootstrap random search the deuterostome node is supported 90 times out of 100 (Fig. 1). Character reconstruction using ACCTRAN and DELTRAN

(MacClade) indicate that the following characters were present in the common ancestor to the deuterostomes; very probably eggs that were flagellated on the animal pole during oogenesis (Frick and Ruppert 1996, 1997), external fertilization, radial cleavage, enterocoely as the primary means of coelom formation, a bilaterally symmetric body with an unpaired anterior coelom and two paired coelomic cavities, a larval apical sense organ, simple epithelia with an intraepithelial nervous system, a closed Harveyian haemal system and a metanephridial excretory system, pharyngeal pores (for eliminating water and concentrating food), organogenesis by extension and differentiation of the coelomic cavities, and a simple notochord (with vacuolated cells, myofilaments, expanded extracellular matrix and extracellular spaces). Deuterostomes lack chitin and arginine phosphate (they store with creatine phosphate) and monociliated cells are prevalent in the epidermis (Nielsen, 1996).

Although early development is normally useful in establishing phylogenetic relationships, thus far experimental embryology with *Saccoglossus kowalevskii* embryos exemplifies both the close relationship among the deuterostome phyla and the difficulty associated with establishing hemichordate polarities, and ultimately deuterostome relationships. Examining the relationship between the plane of first cleavage and the median plane of the embryo has been useful for establishing phylogenies (Conklin 1905, Colwin and Colwin 1950, 1951, Freeman 1982). Echinoderms become bilaterally symmetric somewhere around mid-gastrulation. Echinoderm blastomeres isolated at the two cell stage almost always develop into a normal pluteus. When one of the two primary blastomeres is stained, the stain corresponds to the right or left, dorsal or ventral, or oblique medial plane of the larvae. This shows that the median plane of symmetry is not related to the first cleavage plane, suggesting that this plane is not determined until after the two cell stage (Horstädius and Wolsky 1936). *S. kowalevskii* first shows overt bilateral symmetry at the post-gastrula stage. Like echinoderms, *S. kowalevskii* blastomeres isolated at the two-cell stage develop into normal larvae. When one blastomere is stained, a lateral half of the larvae is always stained, similar to what is observed in tunicates. This indicates that the median plane of symmetry is set up by the two-cell stage (Colwin and Colwin 1950, 1951). Ascidians establish the median plane prior to first cleavage by a cytoplasmic localization of developmental potential, the yellow crescent. The first cleavage occurs through the yellow crescent and separated blastomeres develop into a form which approximates a lateral half of the tadpole larva (Chabry 1887; Conklin 1905). Experimental embryological observations of the pterobranchs and ptychoderid enteropneusts are needed. To date embryological manipulations with *S. kowalevskii* (Colwin and Colwin 1950, 1951) have revealed no compelling reason to classify enteropneusts closer to the echinoderms or to the chordates.

The differences between the hemichordate stomochord and chordate notochords may indicate intermediate stages in the evolution of the deuterostomes leading to the chordate lineage (Ruppert 1997). Bateson (1886) first noted the similarities of the hemichordate buccal diverticulum (stomochord) with the vertebrate notochord, however the hemichordates were subsequently removed from the chordates and the phylum Hemichordata was erected when the stomochord considered to be a homoplasy of the notochord (Komai 1951; Newell 1952). There are, however, significant positional (located ventral to the dorsal hollow nerve cord and dorsal to the gut), developmental (originating from the middorsal wall of the archenteron), and cytological (vacuolated cells, miofilaments, expanded extracellular matrix and extracellular spaces) similarities between the hemichordate stomochord and the chordate notochord (Balser and Ruppert 1990; Ruppert 1997). The hemichordate stomochord differs in that the cells are arranged in a radial fashion around a central ciliated lumen (Welsch and Storch 1970; Welsch et al. 1987; Godeaux 1991). The hemichordate stomochord functions as a skeletal element which antagonizes the contractile pericardium and results in an increased fluid pressure in the heart and across the glomerular blood sinuses (Wilke 1972; Balser and Ruppert 1990). In *S. kowalevskii*, the contractile peritoneal cells of the mesocoel diverticula anchor to the sheath surrounding the stomochord. As a result, the stomochord may also lend structural support and provide an antagonistic base for muscle contractions used for movement of the proboscis (Balser and Ruppert 1990; Pardos and Benito 1997). Significantly, a gene responsible for notochord formation in chordates (Brachyury-T) is not expressed in the developing 'notochord' in enteropneust worms (Peterson et al. 1999), but instead is expressed in the archenteron invagination region and the stomodeum invagination region of the enteropneust *Ptychodera flava* (Tagawa et al. 1998), similar to what is observed in starfish larvae (Shoguchi et al. 1999).

The presence of both a dorsal and a ventral nerve cord in enteropneusts undermines the requirement of congruence and makes it impossible to discern which may be homologous to the vertebrate central nerve cord (Peterson 1995). Bergström (1997) regards the enteropneusts as protostomian based in part on the circumesophogeal nerve ring but unlike the nervous system of protostomes or chordates, the nervous system in hemichordates is subepidermal (intraepidermal in pterobranchs) comparable to that of echinoderms (starfish nerve rings and radial nerves are also intraepidermal) (Cameron and Mackie 1996). Most integrative and sensory neurons, as well as some motor neurons lie peripherally in the epidermal neural plexus (Bullock 1945). The hemichordate neural tube is restricted to the dorsal collar, consists of motor neurons, some interneurons, and conduction pathways (Cameron and Mackie 1996). Only in a few ptychoderids does the cord possess a continuous hollow cavity. The ventral nerve cord of enteropneusts, especially of ptychoderids is large and in *Saccoglossus* provides the main conductive channel (Cameron and Mackie 1996). Although the dorsal cord in chordates may not have a homologue in the hemichordates, gonadotropin releasing hormone, a neuropeptide, which is known to elicit the release of pituitary gonadotropins in vertebrates, occurs in the nervous system of urochordates (Powell et al. 1996) and in enteropneust worms (Cameron et al. 1999).

Pharyngeal pores are considered a homologous character shared by all deuterostome phyla, although they have subsequently been lost in the echinoderms, except, perhaps, in some extinct taxa (i.e., *Cothurnocystis*) (Jefferies 1986). Isolated cDNA clones for Pax1- and Pax9-related genes from urochordates (*Ciona* and *Halocynthia*) and an enteropneust (*Ptychodera flava*) from gill cDNA libraries reveal that they share a common ancestry. Northern blot, RT-PCR/Southern and in situ hybridization analyses revealed that the urochordate Pax 1/9 genes and enteropneust Pax1/9 gene are expressed in the pharyngeal epithelium of developing gill pores (but not in skeletogenesis) (Ogasawara et al. 1999). Although deuterostome gill slits may be mutually homologous structures, the skeleton associated with enteropneust, cephalochordate, and vertebrate gill slits, however, may not be homologous (Godeaux 1991, Nielsen 1994).

Ritter (1894) described an endostyle in tornaria larvae from the California coast and only later did Garstang (1928) propose that the chordate endostyle was derived from the perioral band of the auricularia (Fig. 5a). Recent observations of the enteropneust *Schizocardium brasiliense* have revealed an endostyle-like organ in the dorsal pharynx (Ruppert et al. 1999) rather than in the ventral pharynx where it occurs in chordates. Iodine^{125} binding however, is not restricted to this endostyle-like structure, but occurs throughout the pharyngeal epithelium (Ruppert, personal communication). To date, there is no evidence for chordate endostyle-specific genes (ie: thyroid transcription factor) in the hemichordates. Regulatory genes uniquely expressed during the development of the stomochord, pharyngeal pores and endostyle were almost certainly present in the common ancestor to all the deuterostome phyla and the morphological similarities warrant a re-examination at molecular, ultrastructural and developmental levels to increase our understanding of their evolutionary origins and diversification.

Modifying Garstang's Auricularia hypothesis

The most popular hypothesis on the origin of the chordates is perhaps Walter Garstang's auricularia hypothesis (1894, 1924). Stated briefly, Garstang's auricularia hypothesis postulates a paedomorphic origin from a common ancestor for the Echinodermata, the Enteropneusta, and the Chordata. This ancestor was bilateral and had the appearance of an auricularia larva in which the sides of the body are outlined by a complete circumoral ciliated ridge (Fig. 5b). At the apical pole was situated a pair of pigmented ectodermal pits, or eye-spots. These pits, together with the anus lay within the dorsal or aboral area circumscribed by the circumoral band. The central nervous system of the pelagic auricularia-like ancestor consisted of a nerve ring, which lay underneath the circumoral ciliated band, and therefore enclosed the apical pits (Garstang 1928).

To achieve the chordate body plan, Garstang imagined the ciliated feeding bands from either side of the auricularia mouth to migrate backwards until they opposed one another on the dorsal midline and fused to form the dorsal cord. The ciliated ridges are considered homologous with the medullary folds of the vertebrates, which have a similar relation to the central nervous system. The auricularia apical pigmented pits are shut off from the exterior by the fusion of the ciliated folds. Later, they enlarge and represent the optic vesicles of the vertebrate embryo. To these features Garstang adds the persistence of the larval adoral band and ventral loop as the beginnings of the peripharyngeal band and endostyle, this muscular larvae "which may be distinguished as 'Notoneuralia', would lack only gill slits and a notochord to transform it into a regular chordate tadpole" (Garstang 1928). In order of appearance the auricularia acquired a dorsal cord, optic vesicles, peripharyngeal band and endostyle, gill slits and notochord en route to the chordates.

The shortcoming of Garstang's ingenious scenario is that he removes the ability to feed from the intermediate animal in the transition from 'Dipleurula' to 'Notoneuralia'. The circumoral ciliated feeding band in present day auricularia larva is constrained to a feeding and locomotory function, and therefore is not free to be coopted for a dorsal migration. Even if the ciliated bands were able to move by displacement via differential growth, the transitory animal would be deleteriously affected because it could no longer orient itself in the water or capture food particles. There are two probable routes to overcoming Garstang's transitory animal's feeding and locomotory problem. First, (as suggested to me by E.E. Ruppert) freeing-up the circumoral ciliated band for a dorsal migration could be achieved by an evolutionary transition from planktotrophy to a lecithotrophic chordate ancestor. Alternatively, the locomotory problem could be overcome if the ancestral animal was a tornaria. The animal's feeding function may have been accommodated by the evolution of feeding gill pores, and its locomotory requirements by the presence of a telotrochal ciliated swimming band. This scenario requires that the gill pores evolve *before* the migration of the circumoral ciliated band (and therefore before the formation of the main nerve cord) rather than after as Garstang (1928) proposed.

Several lines of evidence suggest that the ancestor to the deuterostomes resembled a modern day tornaria (Fig. 5a), much like *Tornaria hubbardi* (Ritter and Davis 1904) (Fig. 5a), and was morphologically more sophisticated than Garstang's ancestral Auricularia. *T. hubbardi* has serially arranged gill pores (and no main nerve cord), a proboscis stomochord (a precursor to the notochord), an apical plate with eye - spots (a precursor to the vertebrate optic nerves), and a perioral feeding band that is continuous with a ciliated band in the ventral pharynx (a precursor to the endostyle) (Fig. 5a). This tornaria - like ancestor had an enteropneust - like morphology in its life history, and this 'adult' animal provided the foundations for the evolution of the chordates. Associated with the serially arranged gill pores of the enteropneust - like ancestor were; atrial podocytes, regionalization of the nervous and blood vascular system, and gonads. Branchiometry in the 'adult' ancestor not only preceded the evolution of metamerism in the chordates. Branchiometry, the presence of serially arranged gill elements, was required for the evolution of metamerism in the chordates. Metamerism evolved in the chordates after they diverged from the enteropneust - like ancestor. Metamerism evolved when the tricoelomate body plan formed septa between adjacent gill complexes (gill pores, atria, podocytes, nervous and blood vascular regions, and gonads). Associated with this arrangement came the evolution of myotomes and a notochord (modified from the stomochord for a locomotory function), and an integrative main nerve cord. The latent evolution of the main nerve cord suggests that it did not develop from a 'larval' feeding band, but instead evolved in complexity from regions of the nerve plexus that innervated the ancestral animals gill complex.

The evolution of the echinoderrm auricularia or bipinnaria requires only that the tornaria - like ancestor lose its telotrochal swimming band, apical plate, apical plate muscle, and serially paired gill pores. The evolution of the calcareous skeleton, pentaradial symmetry, and the water vascular system occurred after the divergence of the echinoderms and the tornaria - like ancestor. At some point along the lineage leading to the echinoderms gill pores were lost (Jeffries (1996) provides convincing evidence for the presence of gill pores in fossil echinoderms).

This cladistic study of deuterostome morphology indicates that the ancestor to the deuterostomes was tornaria - like with an enteropneust - like morphology in its life history. From the tornaria - like morphology evolved the echinoderms. From the enteropneust - like morphology evolved the chordates. This scenario requires that the gill slits develop prior to the formation of the chordate nerve cord, and is corroborated by the presence of gill pores and the absence of a nerve cord in *T. hubbardi* (Ritter 1904). Evolutionary developmental studies of the Enteropneusta will undoubtedly reveal that most chordate features; including gill slits (with a feeding function), endostyle (Ruppert et al 1999), metamerism (which evolved as a simple modification on branchiometry), the main nerve cord (homologous with the nerve plexus that innervates the gill complex, rather than with the collar cord), and notochord (modified from the stomochord for a change in function) have precursors in the Enteropneusta.

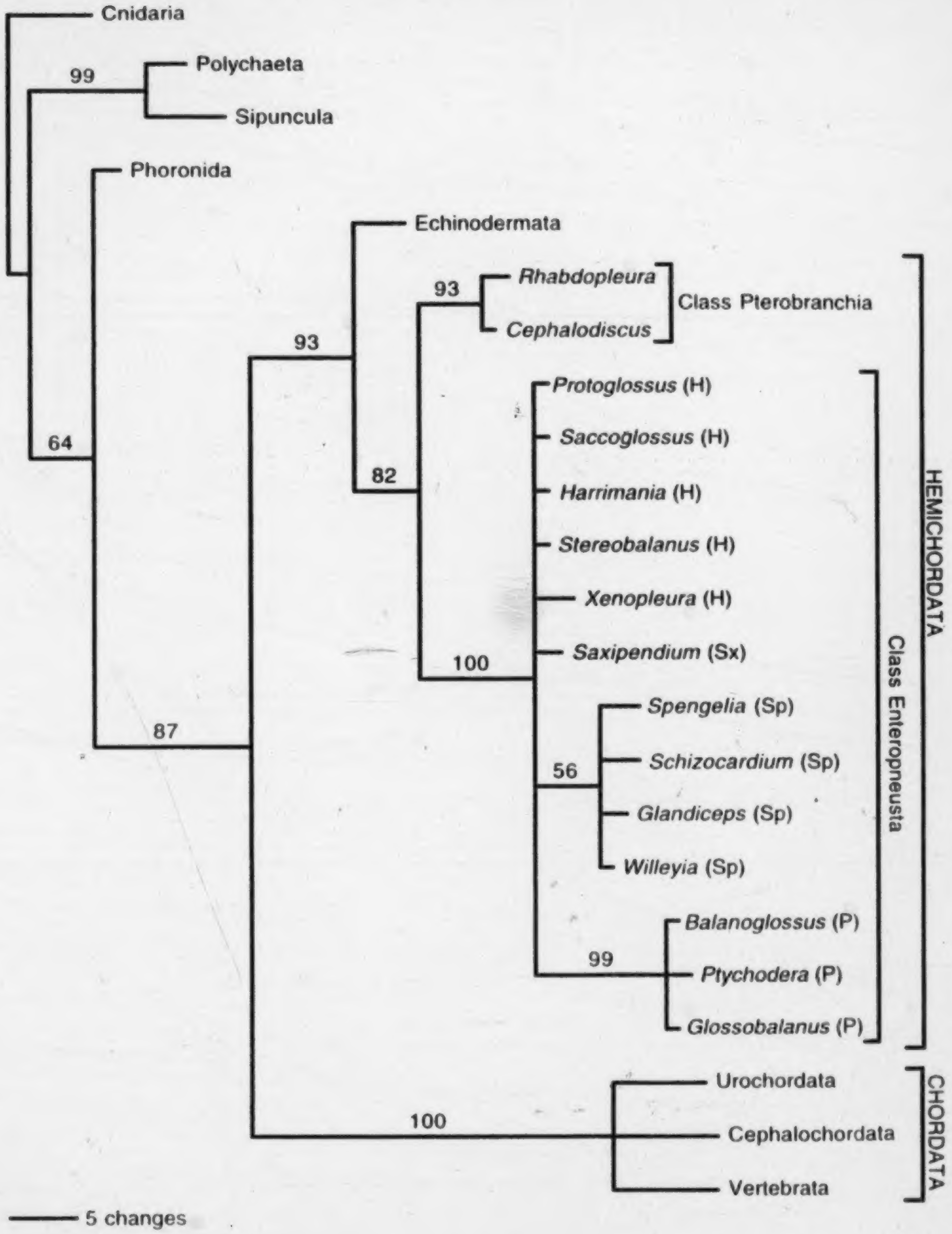
Cnidaria
99
Polychaeta
Sipuncula
Phoronida
Echinodermata
93
Rhabdopleura
Cephalodiscus
Class Pterobranchia
93
Protoglossus (H)
Saccoglossus (H)
Harrimania (H)
Stereobalanus (H)
Xenopleura (H)
Saxipendium (Sx)
100
82
64
Spengelia (Sp)
Schizocardium (Sp)
Glandiceps (Sp)
Willeyia (Sp)
56
87
Balanoglossus (P)
Ptychodera (P)
Glossobalanus (P)
99
Class Enteropneusta
HEMICHORDATA
Urochordata
Cephalochordata
Vertebrata
100
CHORDATA
5 changes

Figure II-1. Bootstrap 50% majority-rule consensus tree obtained with a heuristic search under the assumptions of parsimony (TL 124, CI 0.710, RI 0.791). Numbers adjacent to nodes are bootstrap values based upon 200 replications.

H - Harrimaniidae, Sx - Saxipendidae, Sp - Spengelidae, P - Ptychoderidae.

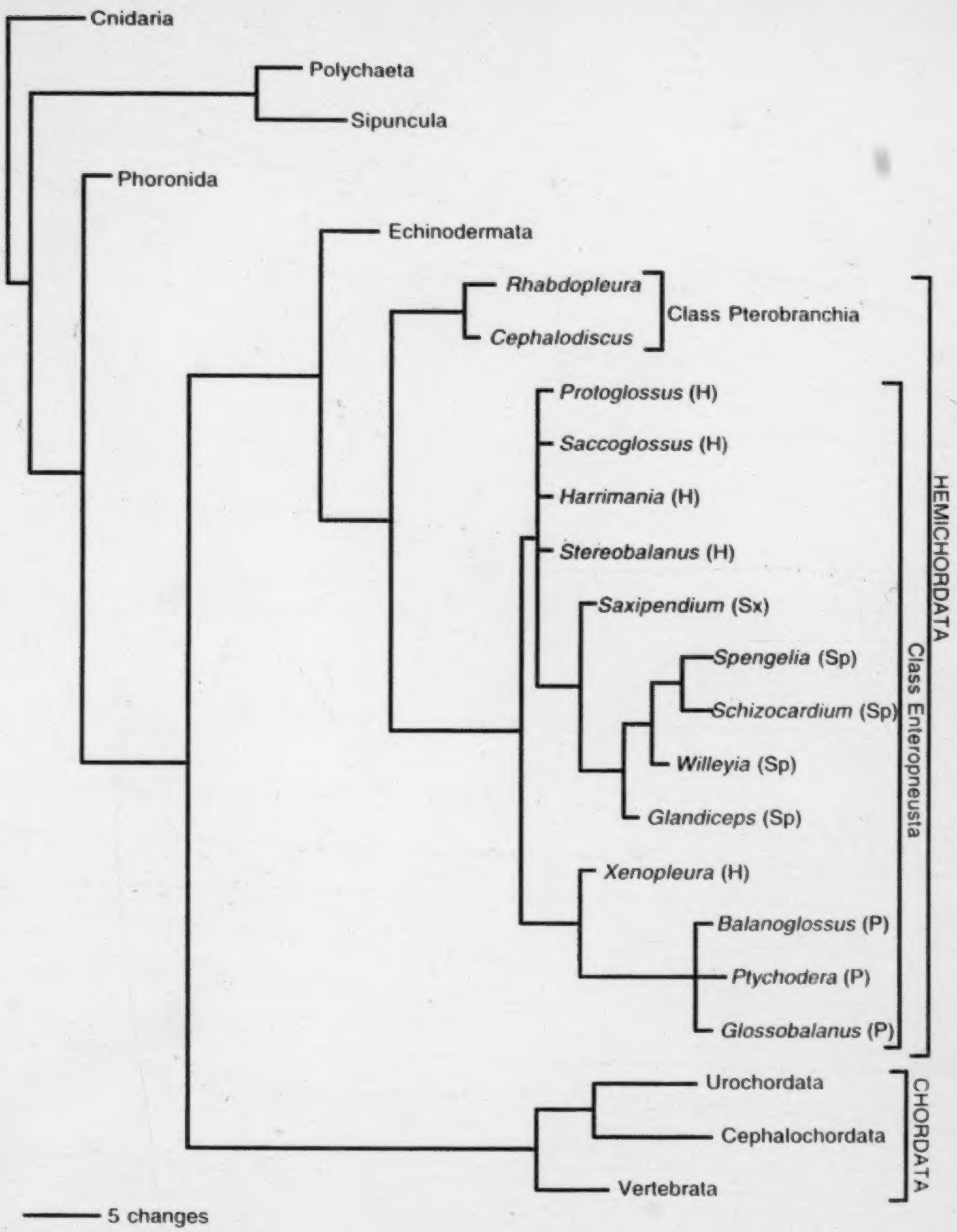
Cnidaria
Polychaeta
Sipuncula
Phoronida
Echinodermata
Rhabdopleura
Cephalodiscus
Class Pterobranchia
Protoglossus (H)
Saccoglossus (H)
Harrimania (H)
Stereobalanus (H)
Saxipendium (Sx)
Spengelia (Sp)
Schizocardium (Sp)
Willeyia (Sp)
Glandiceps (Sp)
Xenopleura (H)
Balanoglossus (P)
Ptychodera (P)
Glossobalanus (P)
Class Enteropneusta
HEMICHORDATA
Urochordata
Cephalochordata
Vertebrata
CHORDATA
5 changes

Figure II-2. One of 6 equally parsimonious trees obtained with a heuristic search, with random stepwise and 200 replications, and TBR branch swapping with MULPARS option on, and steepest descent off. Sum of min. possible lengths = 89, Sum of max. possible lengths = 340, TL 132, CI 0.674, RI 0.829. Family abbreviations as in figure 1.

Figure II-3. Hemichordate anatomy, diagrammatic. A generalized enteropneust. anus (an), atrium (at), dorsal nerve cord (dc), dorsal mesentery (dm), dorsal blood vessel (dv), epidermis (ep), gonad (gd), filtration glomerulus (gl), gill pore (gp), gill slit (gs), heart sinus (hs), liver sacs (ls), mesocoel (mc), paired mesocoel ducts (me), mouth (mo), metacoels (mt), Y-shaped nuchal skeleton (ns), peribuccal coeloms (pb), protocoel (pc), pericardium (pe), ventral post anal extension of the metacoels (pm), pre-oral ciliary organ (poco), protocoel pore (pp), perihaemal coeloms (pr), somatic peritoneum (pt), visceral peritoneum (pt'), septum (sp), stomochord (st), ventral vessel (vv).

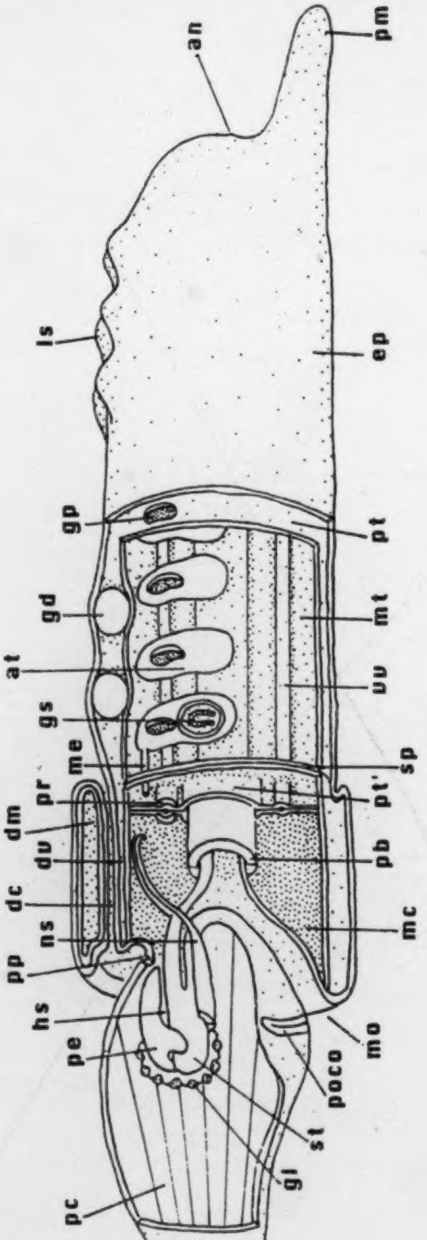
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Figure II-4. Hemichordate anatomy, diagrammatic. The pterobranch *Cephalodiscus*. Pterobranchs are clearly the sister group to the enteropneust worms, though they have a U-shaped digestive tract and ciliated tentacles as adaptations to a tube dwelling life. Abbreviations as in Fig. 3 with the addition of: pigmented band of the cephalic shield (pg), mesosomal arms (ma), stomach (sm), mesosomal tentacles (tn).

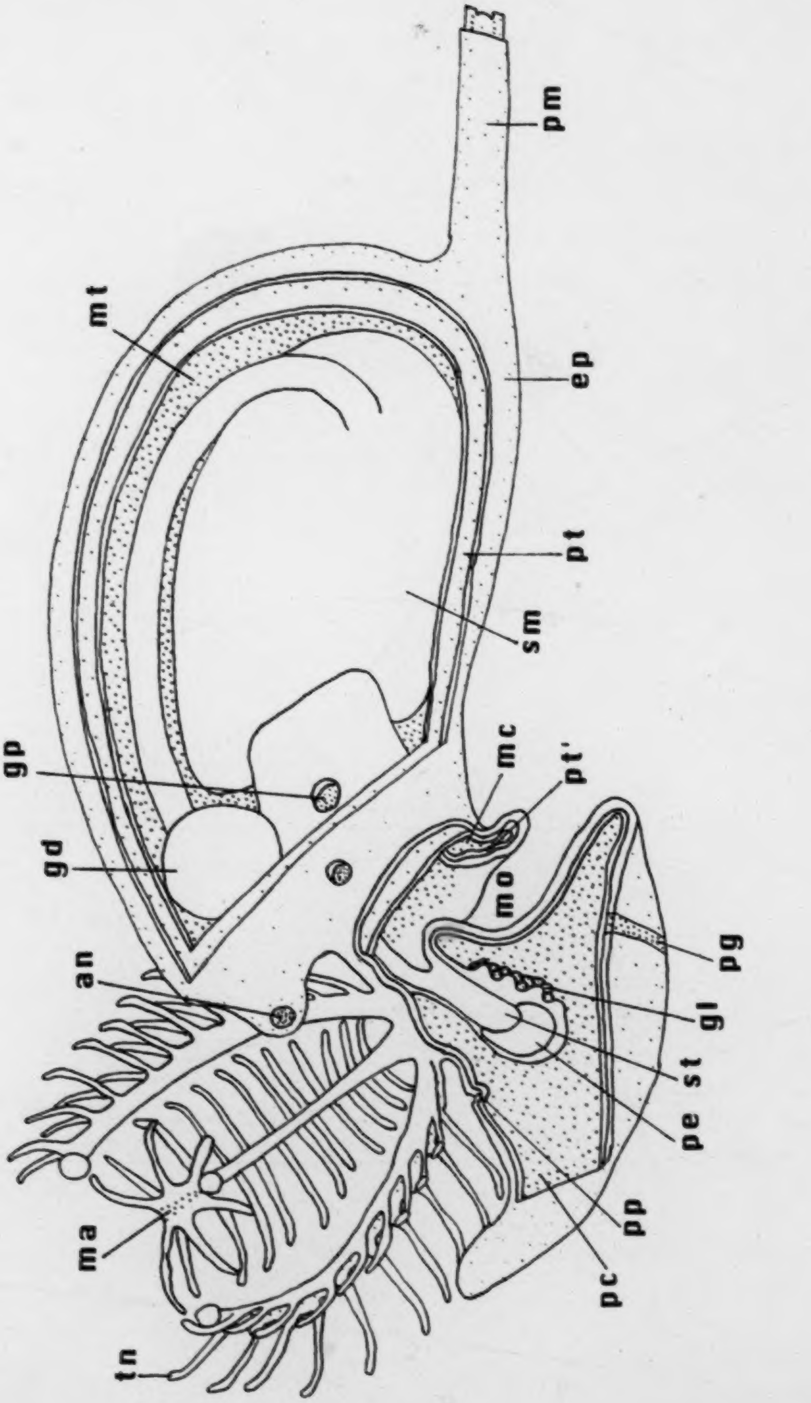

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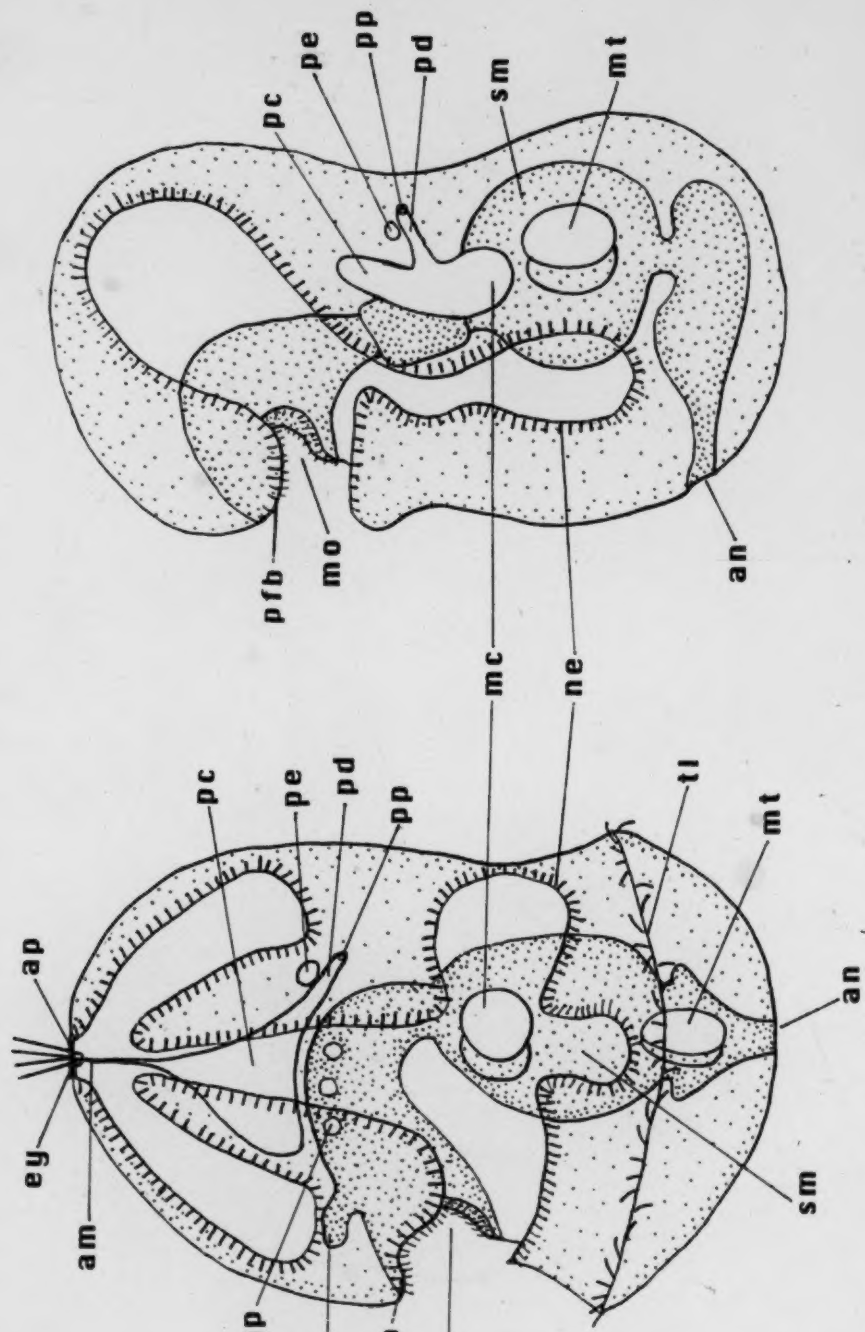
Figure II-5a. This tornaria is redrawn and modified from *Tornaria hubbardi* (from Ritter and Davis 1904) and is the living taxon that most resembles the ancestral deuterostome inferred in this study. *Tornaria hubbardi* has paired eyes, serial paired gill pores and a rudimentary stomochord. There is no evidence for a dorsal nerve cord in the shared ancestor to the deuterostomes. **5b.** This auricularia larva is redrawn and modified from (Gemmill 1914) to demonstrate the homologous characters shared between echinoderm and enteropneust larvae. Abbreviations as in Fig. 3 with the addition of: apical plate (ap), apical plate retractor muscle (am), eye spots (ey), neotroch (ne), perioral feeding band (pfb), stomach (sm), locomotory telotroch (tl).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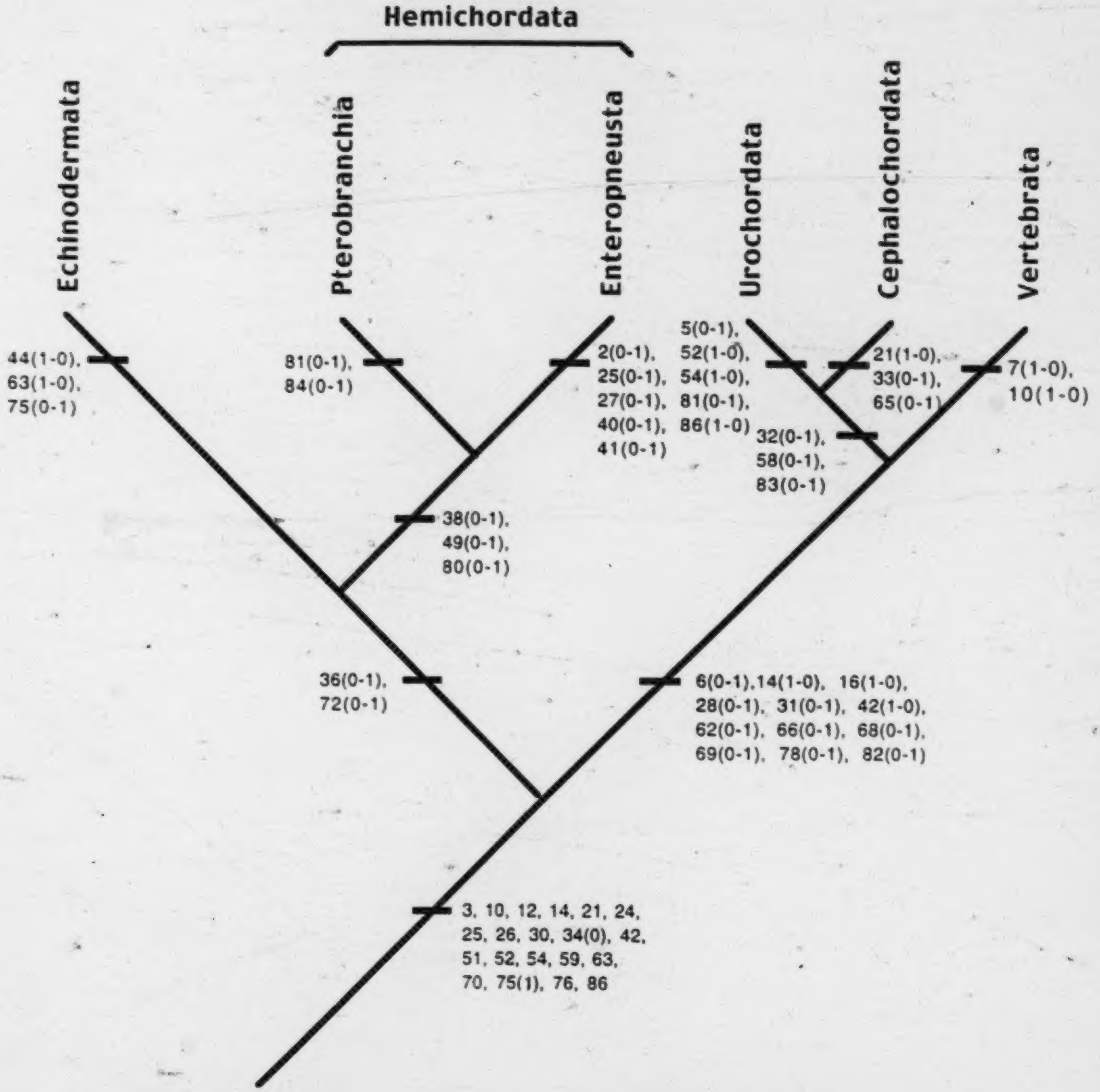
Hemichordata
Echinodermata
Pterobranchia
Enteropneusta
Urochordata
Cephalochordata
Vertebrata
44(1-0), 63(1-0), 75(0-1)
81(0-1), 84(0-1)
2(0-1), 25(0-1), 27(0-1), 40(0-1), 41(0-1)
5(0-1), 52(1-0), 54(1-0), 81(0-1), 86(1-0)
21(1-0), 33(0-1), 65(0-1)
7(1-0), 10(1-0)
32(0-1), 58(0-1), 83(0-1)
38(0-1), 49(0-1), 80(0-1)
36(0-1), 72(0-1)
6(0-1),14(1-0), 16(1-0), 28(0-1), 31(0-1), 42(1-0), 62(0-1), 66(0-1), 68(0-1), 69(0-1), 78(0-1), 82(0-1)
3, 10, 12, 14, 21, 24, 25, 26, 30, 34(0), 42, 51, 52, 54, 59, 63, 70, 75(1), 76, 86

Figure II-6. An unrooted tree of the deuterostomes with the characters and hypothesized character states at the base of the tree. All character states at the base are 1 (present), with the exception of characters 34 (state = 0) (notochord is anterior to the gut), and 75 (state = 1) (closed Harveyian haemal system). Character numbers at the internodes are accompanied by the state changes (in parenthesis). The character numbers correspond with the character numbers in Appendix 1.

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Appendix II-1.

List of Morphological Characters Unless otherwise stated, all character states are [0 = absent, 1 = present, P = polymorphic (0 and 1), ? = unknown].

1] egg barrier: [0 = fertilization membrane, 1 = chorion].

2] yolk + egg cells: echinoids and enteropneusts have cells in the germinal epithelium that are specialized for yolk synthesis. The synthesized yolk is then transported to the developing egg (ie: the egg is not responsible for yolk synthesis).

3] radial cleavage

4] spiral cleavage

5] strict cell fate determination [0 = no, 1 = yes]

6] primary blastomere separation: [0 = complete larvae; 1 = approximates a half larvae]. (Chabry 1887 and Conklin 1905 for tunicates; Conklin 1933 for cephalochordates; and Colwin and Colwin 1950 for hemichordates).

7] polar bodies: [0 = oral pole, 1 = apical pole]

8] mesoderm is derived from the embryonic 4D cell (a protostome feature)

9] blastopore becomes the mouth

10] blastopore becomes the anus

11] coelom via schizocoely

12] coelom from archenteron

13] unpaired anterior enterocoel

14] trimery, a body arranged around three coelomic cavities

15] unpaired anterior coelom lined with podocytes [0 = no, 1 = yes]

16] paired anterior coelomopore: derived from somatic ectoderm (Welsch and Welsch 1978; Balser and Ruppert 1990).

17] paired mesocoels

18] paired metacoels

19] pericardial coelom

20] gonocoel

21] approximate bilateral symmetry: Jefferies (1986; 1990). Jefferies argues that the common ancestor of echinoderms and chordates underwent dexiothetism, resulting in a left-side bias in recent echinoderms and chordates, as well as carpoids.

22] monociliated cells: enteropneusts have monociliated cells in the larval neotroch (Nielsen 1987)

23] multiciliated cells: enteropneusts have multiciliated cells in the proboscis, stomochord, epithelia, archenteron and epidermis.

24] neotroch: larval multiciliated feeding band.

25] telotroch: larval monociliated locomotory band.

26] larvae with apical sense organ

27] larval apical muscle band

28] unpaired pineal and parietal eyes

29] peripharyngeal bands: bands on the inner pharynx of urochordates.

30] notochord has vacuoles: notochord is defined here as a middorsal outfold of endodermal gut; turgid cells bear a central vacuole (Ruppert 1997). All notochords have a non-cellular fibrillar sheath made of collagen. "Significant positional (located ventral to the dorsal hollow nerve cord and dorsal to the gut), developmental (originating from the middorsal wall of archenteron), and cytological (vacuolated cells, myofilimants, expanded extracellular matrix) similarities exist between the hemichordate stomochord and the chordate notochord. The differences between the stomochord and established notochords may be indicative of intermediate evolutionary stages and the similarities perhaps warrant a re-examination of the evolutionary importance of the stomochord with respect to the notochord." (Ruppert 1997 p. 245) [0 = no, 1 = yes]

31] notochord has discoid chordal cells: cells arranged in a single file with intercellular spaces occur between cells (Ruppert 1997) [0 = no, 1 = yes]

32] notochord has extracellular spaces [0 = no, 1 = yes]

33] notochord has transverse myofilaments [0 = no, 1 = yes]

34] notochord position; [0 = anterior to gut, 1 = coextensive with gut]

35] notochord has vermiform process: a dorso-ventraly flattened anterior extension of the stomochord. [0 = no, 1 = yes]

36] heart has glomerulus: the hemichordate complex (Dilly et al. 1996) is believed to be homologous to the echinoderm axial complex as both consist of a protocoelomic wall of a large blood vessel with a zone of podocytes where primary urine is formed by ultrafiltration of blood from the haemal system to the protocoel (Nielsen 1996; Balser and Ruppert 1997) and is hypomere derived (Balinsky 1981; Uribe 1992). [0 = no, 1 = yes]

37] heart has diverticula [0 = no, 1 = yes]

38] mucociliary pre-oral locomotory organ

39] cauliflower organ: (or racemose organ) ventrolateral compartments of the proboscis coelom that are prolonged into the proboscis stalk where their walls are greatly saculated (Hyman 1959).

40] Pre-oral ciliary organ: a horseshoe shaped ciliated band on the ventral posterior proboscis (Brambell and Cole 1939).

41] nuchal skeleton: a Y - shaped collagenous structure running between the protocoel and mesocoel.

42] subepithelial nervous system: collar cord of enterpneusts is not homologous to the dorsal cord of chordates (Cameron and Mackie1996).

43] paired and fused ventral nerve cord

44] dorsal nerve cord

45] neurulation: the invagination of a subepidermal nerve plexus to form a cord.

46] nerve cord lacunae: [0 = absent sporatic lacunae, 1 = continuous canal]

47] neuropore: [0 = anterior and posterior, 1 = anterior only] modified as Kolliker's pit in adult cephalochordates (Tjoa and Welsch 1974).

48] anterior to posterior flow in neurocoel

49] paired valved collar ducts: ducts leading from the collar coeloms to the outside (either directly or via the first pair of gill atria) in enteropneusts.

50] lateral trunk septa: gonadal wings of some enteropneuts that are segmented by a transverse septa separating the dorsal side from the ventral side.

51] pharyngeal pores: Godeaux (1974) believed the gill slits of enteropneusts and cephalochordates to be homoplasy.

52] gill slits have tongue bars (secondary bars of gills): consequence of U-shape. [0 = no, 1 = yes]

53] peripharyngeal diverticula: trunk coelom diverticula that extends into the tongue bars.

54] synapticles: cross connections which bound the primary and secondary gill bars resulting in fixed and immovable secondary bars.

55] atrium arrangement: [0 = serially arranged, 1 = continuous]

56] podocytes

57] branchiomeric podocytes

58] gametes released into atrium

59] gonads with separate gonoducts

60] gametes pass through coelom and out a common gonadopore.

61] genital ridge

62] endostyle: a specialized groove of pharyngeal endoderm that secretes mucoid material that is generally propelled dorsally and caudally by pharyngeal cilia to trap food. Iodine affinity is further evidence of homology (Barrington 1965).

63] iodotyrosine secretion

64] pygochord: a midventral band of vacuolated cells in the trunk (Spengel 1893; Uribe 1992).

65] post-branchial chamber: an extension of the dorsal chamber of the pharynx lacking gill slits is characteristic of the Spengelidae (Barrington 1940).

66] hepatic diverticula (liver sacs): paired digestive caeca that can be seen from the exterior that are commonly more pigmented than other epithelia.

67] location of circular muscles with respect to longitudinal muscles: [0 = external, 1 = internal]

68] dorsal postanal tail

69] segmented longitudinal muscle arrangement: developed from rows of mesodermal pockets from the archenteron.

70] dorsal trunk vessel

71] dorsal vessel blood flow: [0 = anterior, 1 = posterior]

72] central sinus: a blood sinus that is normally associated with a pericardium and cells involved in ultrafiltration.

73] peribuccal fold: coelomic invasion into the collar from the trunk forming a narrow coelomic space around the buccal epithelium, generally filled with circular muscle fibers (Hyman 1959).

74] perihaemal diverticula: trunk coelom diverticula that extends through the collar into the neck (different than pericardium or heart coelomic cavity) that parallels the dorsal blood vessel.

75] haemal system: [0 = none, 1 = closed Harveyian, 2 = closed Galenic], Galenic flow is tidal and Harveyan flow is circuitous. Vertebrates, for example, have Harveyan flow, asteroids probably Galenic (mostly) and phoronids, including the lophophore, a combination of the two (because blood flow is tidal in each tentacle). Galen was a Greek physician who thought that human blood flow was tidal and William Harvey was a 17th century anatomist and doctor who proved otherwise.

76] metanephridial system

77] protonephridia

78] lymphocytes

79] hemerythrin

80] ventral locomotory-adhesive post-anal tail: the stolon of pterobranchs is considered homologous to the ventral post anal tail of enteropneust juveniles. Jeffries (1986) argues for homology between the tail of chordates, the stalk of hemichordates (and the aulacophores of carpoids).

81] U - shaped gut

82] epipharyngeal groove in intestine

83] inhalent and exhalent siphons

84] red band: a pigmented band of cells on the posterior cephalic shield of pterobranchs.

85] chitin synthesis

86] sialic acid

Appendix II-2

Morphological Data Matrix;

	11111111112222222222333333333344444444445555555555666666666677777777778888888
Taxon	12345678901234567890123456789012345678901234567890123456789012345678901234567890123456
Cnidaria	0?0000001000????????01000000???????0000001000?????0???????0?0000?0??0??00000000?000010
Sipuncula	0?011011101010000001101001000??????0000000100???000????1??010000?00000?000?1?010100000
Polychaeta	0?011011101010001101101001000??????00?0001100???000????1??01?000??00110000?11010000010
Phoronida	0?100010011011000100110001000??????0000001000???000????1??010000?000010000P11000100010
Echinodermata	00100010010111111110110101000??????1000001000???001????0??100000?00001?100210000000001
Rhabdopleura	00100?100101110111101101010001000001010001010???100???????10?0?0?000010100110001100101
Cephalodiscus	00100?100101110111101101010001000001010001010???1010????0010?0?0?000010100110001100101
H-Protoglossus	01100?10010111111110111111100100000101011101111010110001101000?10000001010011000100000 01
H-Saccoglossus	01100010010111111110111111100100000101011101111010110001101000?100000010100110001000001
H-Harrimania	01100?10010111111110111111100100000101011101111010110001101000?100000010100110001000001
H-Stereobalanus	01100?101011111111011111110010000010101110111101011100?110100?100000010100110001000001
H-Xenopleura	01100?10010111111110111111100100000101011101110010110001101000?10?000010101110001000001
Sx-Saxipendium	01100?1001011111111011111110010000011?101110111?01011?00110100?1000000101??110001000001
Sp-Spengelia	01100?10010111111110111111100100001111011101111010 11?10110100?10?01001011?110001000001
Sp-Schizocardium	01100?100101111111101111111001000011110111011110101101011010 1?100110010110110001000001
Sp-Glandiceps	01100?100101111111101111111001000011110111011110101100011010 1?10?010010100110001000001
Sp-Willeyiaa	01100?1001011111111011111110010000 11?101110111101011?00110101?10??1001011?110001000001
P-Balanoglossus	01100?1001011111111011111110010000010111110111001111101101 01?1P110001011111000100000 1
P-Ptychodera	01100?1001011111111011111110010000010111110112001111101101 01?1P110001011111000100000 1
P-Glossobalanus	01100?1001011111111011111110010000010111110111001111101101 01?1P110001011111000100000 1
Urochordata	1010111001?10000001010100101111101000000000011211??10?01?011?01100101?1?000110100111000
Cephalochordata	1010011001011000??00010001011111110000000000112110011011?11100110110111100011010001 1001
Craniata	1010010001110000??0010100101111001000000000011211001101P000100110010111100011010001 0001

Chapter III

Evolution of the Chordate Body Plan: New Insights from Hemichordate Phylogeny*

Abstract Hemichordates include two distinct groups, the enteropneust worms and the colonial pterobranchs. Most hypotheses about the origin of the chordates have assumed that extant colonial pterobranchs represent the primitive body plan, with enteropneusts being derived hemichordates. We present a molecular phylogenetic analysis of the Hemichordates that challenges this long - held view. We used 18S rRNA to assess relationships within the hemichordate classes Pterobranchia and Enteropneusta. Phylogenetic tree topologies were inferred with PAUP using parsimony, maximum likelihood and distance methods. This is the first phylogenetic study of the deuterostomes to include several hemichordates. Unlike previous studies we found that the enteropneust worms segregated into two distinct clades, the morphologically complex Ptychoderidae, and the relatively simple Harrimaniidae. Surprisingly, the pterobranch *Cephalodiscus* fell within the harrimaniid clade rather than aligning as an outgroup to the enteropneusts, as previously suggested by morphological cladistic analysis. Unfortunately, 18S rDNA analysis cannot resolve the deep deuterostome clades, so the position of the hemichordates within the deuterstomes remains unresolved. The nesting of pterobranchs within the Enteropneusta suggests that enteropneust worms are basal hemichordates thus displacing the pterobranchs from their traditional basal position in the phylogeny of the Deuterostomia. The possibility that Enteropneusta is the primitive clade of deuterostomes has profound implications for models of deuterostome, and chordate evolution.

*This chapter has been modified and submitted to Proceedings of the National Academy of Sciences (USA) as: Christopher B. Cameron, James R. Garey[°] and Billie J. Swalla[*] 2000. Evolution of the Chordate Body Plan: New Insights from Phylogenetic Analyses of Deuterostome Phyla.

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Introduction

Phylogenetic relations among the Deuterostomia, and debates over the ancestor to the Chordata have challenged zoologists throughout the 20th century because of high morphological disparity among taxa, a poor fossil record for the soft-bodied hemichordates and invertebrate chordates, and a paucity of modern research on hemichordates. The last two decades of the 19th century saw an intensive study of the Enteropneusta, due in a large part to Spengel (1). When Metschnikoff (2) discovered that tornaria larvae were larvae of enteropneusts rather than of echinoderms, the shared ancestry of the enteropneusts and echinoderms was generally assumed. Bateson (3), on the other hand, believed that the enteropneusts were more closely related to the chordates, based on his studies of their embryology and on the presence of gill slits and a partial notochord.

When the first member of the class Pterobranchia, *Rhabdopleura,* was dredged from the deep sea by Sars (4), pterobranchs were thought to be related to Bryozoa. Pterobranchs are colonial, live in secreted tubular coenecia and reproduce via a short - lived planula-shaped larvae or by asexual budding. When *Cephalodiscus* was discovered it was immediately recognized to be related to *Rhabdopleura*, and together they were thought to be related to the Bryozoa and Phoronida, with whom they shared lophophore-like ciliated feeding tentacles (4). Fowler (5) recognized the importance of the gill slits as a synapomorphy of the Enteropneusta and *Cephalodiscus* and Willey (6) divided the hemichordates into two classes; Enteropneusta and Pterobranchia. The third extant class of hemichordate, the monotypic Planctosphaeroidea (7), is known only as larvae.

Planctosphaera pelagica is a large gelatinous larva that collects food with an extensive array of ciliated bands, and may represent a tornaria modified by hypertrophy due to a long planktonic life (8). *P. pelagica* is not represented in this study.

Hemichordates have been considered a sister group to echinoderms (4, 7, 9, 10, 11) to cephalochordates (12), and to chordates (13). Recent cladistic analysis of morphological data sets have revealed that the pterobranchs are basal deuterostomes, whereas enteropneusts are an early offshoot of the chordate lineage, suggesting that the phylum Hemichordata is polyphyletic (13, 14). Molecular and morphological analyses of axis specification suggests that the enteropneusts may be inverted dorso-ventrally with respect to protostomes (15, 16, 17, 18). Previous studies employing 18S rRNA sequences to construct a deuterostome phylogeny with the enteropneust *Saccoglossus kowalevskii* (19, 20) and partial gene sequence (21) find support for a hemichordate-echinoderm clade and weak support for a hemichordate-chordate clade, but do not address evolution within the Hemichordata. Given the wide range of hypotheses of deuterostome relations, and the limited information for hemichordates, we embarked on a project to assess relations among the hemichordates, expanding the array of complete18S rDNA sequence for hemichordates and adding three new ascidian sequences.

Materials and Methods

Animals. Cephalodiscus gracilis and *Ptychodera bahamensis* were collected from Bermuda and stored in 70% ethanol. *Saccoglossus* species A and *Harrimania planktophilus* were collected subtidally from Barkley Sound, British Columbia (Table 1).

Sequences. The 18S ribosomal gene was chosen because of the large number of sequences available in databases (GenBank) for representative deuterostomes (Table 1) and because it has been useful for resolving urochordate relations (22). New sequences were obtained as previously described (23) with subsequent ABI Prism Dye Terminator Ready Reaction automatic sequencing (Perkin Elmer) run on a Perkin Elmer 377 autosequencer. Primers used for PCR amplification and subsequent DNA sequencing were

18SA 5'-CAGCAGCGCGGTAATTCCAGCTC-3' and 18SB 5'-AAAGGGCAGGGACGTAATCAACG-3' (24, 25). Additional primers included 18SC 5'-TTAGAGTGTTCAAAGCAGGC-3', 18SD 5'-CGATCAGATACCGTCCTAGT-3', 18SE 5'-CGTTCTTAGTTGGTGGAGCG -3', 18SF 5'-GCCTGCTTTGAACACTCTAA-3', 18SG 5'-ACTAGGACGGTATCTGATCG-3', 18SH 5'-CGCTCCACCAACTAAGAACG-3'.

Alignments and Analysis. Sequences were aligned with Clustal V (26) at the order and phylum level first and then were hand aligned according to the secondary structure model of the eukaryotic small ribosomal subunit (27). Phylogenies were inferred using maximum parsimony and neighbor-joining in PAUP (28). Maximum parsimony with PAUP used heuristic searches with stepwise random addition of taxa and TBR branch swapping with the MULPARS option in effect, and collapsed zero-length branches. Neighbor-joining trees were constructed using Kimura's two-parameter distances. Analyses were done by excluding variable regions with ambiguous alignment and also by including all data with no exclusion. No differences in tree topology were observed between the excluded versus non-excluded analyses thus all topologies presented are using non-excluded data sets

Results

Relationships within the Deuterostome Taxa. We obtained strong support for four distinct monophyletic deuterostome clades: the echinoderms, the hemichordates, the urochordates, and the vertebrates plus cephalochordates. However, relationships among the deuterostomes were difficult to discern with this dataset presumably due to unequal rates of evolution. Neighbor-joining analyses indicated that the monophyletic Hemichordata are sister taxon to the echinoderms (Fig. 1B). Two protostome species were used as outgroups for the analysis, an annelid (*Nephtys hombergi*) and an arthropod (*Tenebrio molitor*). Further data are needed to determine relationships within the deuterostomes clearly.

Relationships within the Hemichordates. All of the trees obtained by any of the phylogenetic methods showed strong support for the monophyly of the hemichordates (Fig. 1). Furthermore, the enteropneust worms consistently formed two clades, also with high bootstrap support. These two clades correspond to two generally accepted families, the large and complex worms in the Ptychoderidae, and the relatively small and simple animals of the Harrimaniidae. We obtained several different taxa within each family in an effort to improve the phylogenetic signal. Surprisingly, the colonial pterobranch included in the study, *Cephalodiscus gracilis*, fell in one of the two enteropneust clades, as the sister taxon to the harrimaniid worms (Fig. 1).

Discussion

Deuterostome Phylogeny. We found the Vertebrata to be monophyletic and sister taxon to the Cephalochordata, as suggested by previous studies (29). The Urochordata was also monophyletic as shown previously (22), however its relationship to other deuterostomes remains poorly resolved. This study found strong support for hemichordate monophyly, and for an echinoderm + hemichordate clade. The monophyly of pterobranch and enteropneust hemichordates is supported morphologically by the shared presence of a muscular-secretory-locomotory pre-oral proboscis which encompasses a heart / kidney coelomic complex (34, 35 (for *Cephalodiscus*), 36 (*Glossobalanus*), 37 (*Saccoglossus*)), paired valved mesocoel ducts and pores (34), and a ventral post anal extension of the metasome (4,7).

In this study the echinoderm + hemichordate clade was strongly supported as suggested by morphological evidence. For many years after its discovery the enteropneust tornaria was considered to be the larva of an echinoderm, in particular an auricularia or bipinnaria of an asteroid. These large gelatinous larvae share a preoral larval feeding band that creates an upstream feeding current using monociliated cells (30, 31) and a perioral ciliated band that manipulates food into the esophagus. Although there are many gaps in our knowledge of coelomogenesis in the hemichordates, and what is known is sometimes interpreted from a limited set of ontogenetic stages, the detailed organization of the coelomic sacs nevertheless provides a convincing comparative test of their sister-group status. The coelomic sacs in hemichordates and echinoderms are organized anterior to posterior in three regions: the unpaired protocoel (echinoderm axocoel), paired mesocoels (echinoderm hydrocoels) and metacoels (echinoderm somatocoels). Development of the coeloms is primarily enterocoelous although deviation from the ancestral norm is seen in both phyla (32). For an extensive comparison of echinoderm larvae to tornaria larvae see (30, 33, 41).

Colonial Pterobranchs likely evolved from solitary Enteropneusts. In this study we obtained 18S rDNA sequences from a diverse assemblage of hemichordates including *Cephalodiscus* from the class Pterobranchia and 4 acorn worms (class Enteropneusta) in an effort to construct a robust molecular phylogeny with particular emphasis on hemichordate relationships. Prevailing hypotheses suggested that the class Pterobranchia, represented by *Cephalodiscus* and *Rhabdopleura*, were either basal deuterostomes (11) or plesiomorphic hemichordates (4). This 18S rDNA study indicates that the pterobranchs are instead derived from within one enteropneust clade (Fig. 1). This is the only tree topology of echinoderms, pterobranchs, enteropneusts plus chordates that has not been suggested by other authors, so the high bootstrap support for such a topology is most surprising.

The hemichordate family Harrimaniidae (class Enteropneusta) + class Pterobranchia do not possess the morphological complexities of the ptychoderids, they have no hepatic sacs, and no genital wings. *Cephalodiscus* has only a single pair of clefts in the pharynx without gill skeletal bars. Most enteropneusts, on the other hand, have collagenous gill bars framing the pores in the pharyngeal epithelium, extending the pores dorso-ventrally into elongated slits. In harrimaniids the bars are simple, with no synapticles and in some cases (the genus *Stereobalanus*) no secondary bars in the gill slits. The genus *Protoglossus* has short primary bars, and in all enteropneust juvenile worms the gill bars are not yet developed, and at this stage most resemble the pores of *Cephalodiscus*. *Stereobalanus* has no skeletal elements for all of the 'slits' are joined by a single pore forming an open channel.

If this topology is correct, then pterobranchs are unlikely to be the plesiomorphic taxon of hemichordates, but rather may have evolved from an enteropneust-like ancestor, perhaps in relation to an evolutionary decrease in body size and the adaptation of a tubicolous, filter-feeding habit. Morphological tests of this hypothesis may be sought in the evolutionary, developmental, and functional morphology of harrimaniid enteropneusts. An enteropneust-to-pterobranch evolutionary transition may be forshadowed in enteropneusts by:

1) Developmental evidence of a posteroventral outgrowth of the metasome (stalk, stolon) of juvenile *Saccoglossus* and *Harrimania*.
2) Functional evidence of filter feeding in *Harrimania planktophilus* (chapter VII).
3) Heterochronic trend towards body size reduction.
4) Occurrence of two, rather than one, protocoel duct and pore.
5) Reduction in the number and complexity of gill pores.
6) Reduction and disappearance of atria and coelomic diverticula (perihaemal, peripharyngeal and peribuccal coeloms of *Stereobalanus* and *Protoglossus*).
7) Reduction in the number of cilia / cell and number of gonads?
8) Loss of larval telotroch?
9) Reduction of nuchal skeleton?

Although comparative data are limited at present, the foregoing discussion suggests that the hypothesis of an enteropneust to pterobranch transition is plausible and merits further investigation, particularly in genera such as *Stereobalanus* and *Protoglossus*.

Autapomorphies of the family Ptychoderidae (class Enteropneusta) including *Ptychodera, Balanoglossus* (Fig. 1) and *Glossobalanus* (not included in this study) are paired dorsolateral wings of the anterior trunk that house the gonads, anteriorly projecting coelomic diverticula (38), hepatic sacs and well developed gill-slit skeletal bars. Ptychoderids typically develop via a tornaria. The most parsimonious tree obtained with a maximum likelihood search (Fig. 1) revealed the family Ptychoderidae as monophyletic and sister group to family Harrimaniidae + class Pterobranchia (*Cephalodiscus*). In ptychoderids, the gill-slit skeletal bars are extensive, with a secondary bar separating the original cleft down the midline, creating essentially a pair of slits which share a common atrium to an outer pore. Ptychoderids have synapticles, or supporting cross bars that run horizonatally joining primary bars to the secondary (or 'tongue') bar that support the bars from moving freely.

Implications for Evolution of the Chordate Body Plan. The placement of the Pterobranchia (Fig. 1A & B) suggests that the common ancestor to the deuterostomes was not a lophophore-like organism. Given that adult Enteropneusta exhibit some chordate characteristics, namely pharyngeal gill pores, an endoderm-derived stomochord (which has notable similarities with the chordate notochord) (4, 37), and an endostyle-like structure in the pharynx (39), whereas the echinoderms do not (although see 11), it seems most parsimonious to consider an enteropneust-like ancestor from which the Echinodermata (along one lineage) and the Chordata (along another) evolved. There is some molecular evidence that the branchial basket of ascidians and the gill slits of enteropneusts share a common ancestor. When expression patterns of Pax1- and Pax9-related genes from urochordates (*Ciona* and *Halocynthia*) and an enteropneust (*Ptychodera flava*) were compared by in situ hybridization, the urochordate Pax 1/9 genes and enteropneust Pax1/9 gene are expressed in the pharyngeal epithelium of developing gill pores in both phyla (40) suggesting that the pores are homologous. Pax9 does not appear to have a role in enteropneust skeletogenesis.

A detailed analysis of the development of the stomochord, dorsal hollow collar nerve and pharynx of enteropneust worms may provide insight into the evolutionary origin of these structures. Hemichordates lack a postanal tail, and segmentation of the major functional systems, such as the muscular and nervous systems (4) characteristic of chordates (but not in urochordates). These results in turn may then allow an understanding of how the evolutionarily successful chordate body plan first originated.

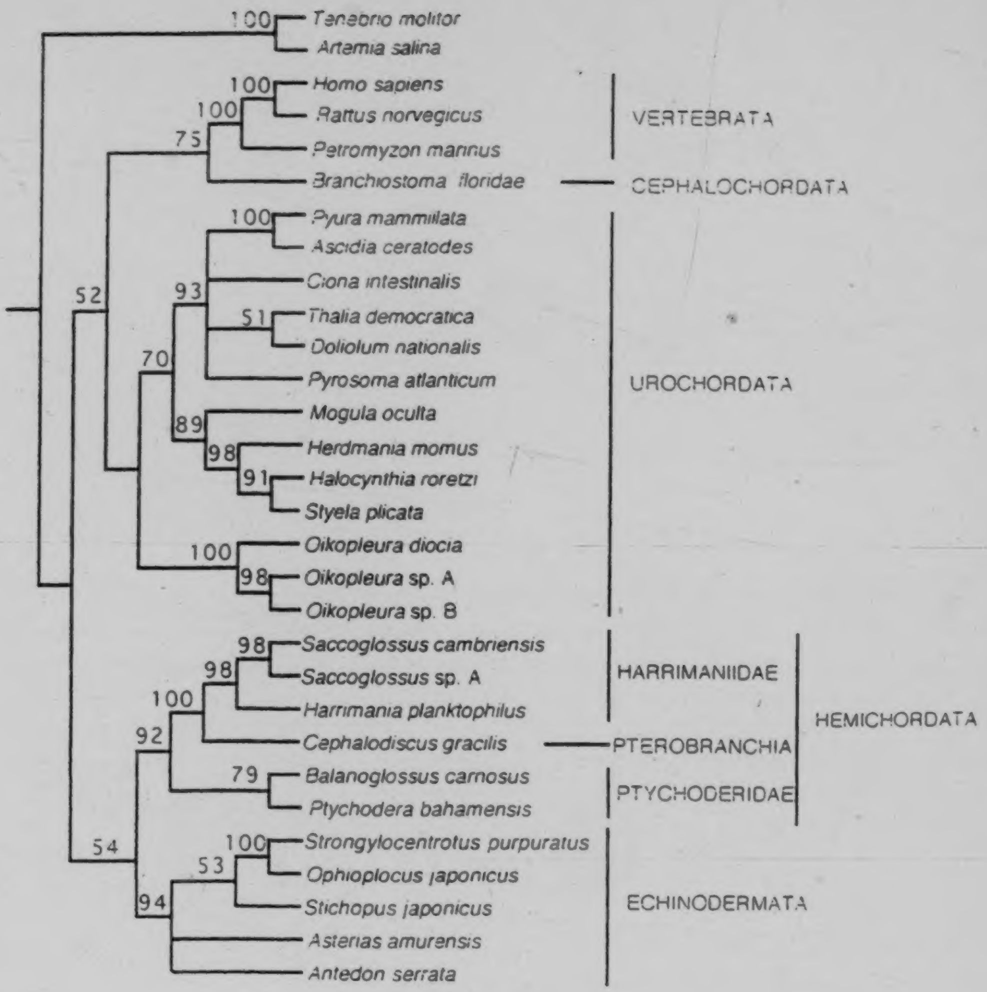
100
Tenebrio molitor
Artemia salina
100
Homo sapiens
100
Rattus norvegicus
75
Petromyzon marinus
Branchiostoma floridae
VERTEBRATA
CEPHALOCHORDATA
100
Pyura mammillata
Ascidia ceratodes
52
93
Ciona intestinalis
51
Thalia democratica
Doliolum nationalis
70
Pyrosoma atlanticum
Mogula oculta
89
98
Herdmania momus
91
Halocynthia roretzi
Styela plicata
UROCHORDATA
100
Oikopleura diocia
98
Oikopleura sp. A
Oikopleura sp. B
98
Saccoglossus cambriensis
98
Saccoglossus sp. A
100
Harrimania planktophilus
92
Cephalodiscus gracilis
79
Balanoglossus carnosus
Ptychodera bahamensis
HARRIMANIIDAE
PTEROBRANCHIA
PTYCHODERIDAE
HEMICHORDATA
54
100
Strongylocentrotus purpuratus
53
Ophioplocus japonicus
94
Stichopus japonicus
Asterias amurensis
Antedon serrata
ECHINODERMATA

Figure III-1A. Phylogenetic tree illustrating evolutionary relationships among deuterostome taxa as inferred from 18S rDNA sequence analysis, and constructed with maximum parsimony using heuristic searches with stepwise random addition of taxa and TBR branch swapping with the MULPARS option in effect, and collapsed zero-length branches. Numbers above branches indicate bootstrap percentages based on 200 replications.

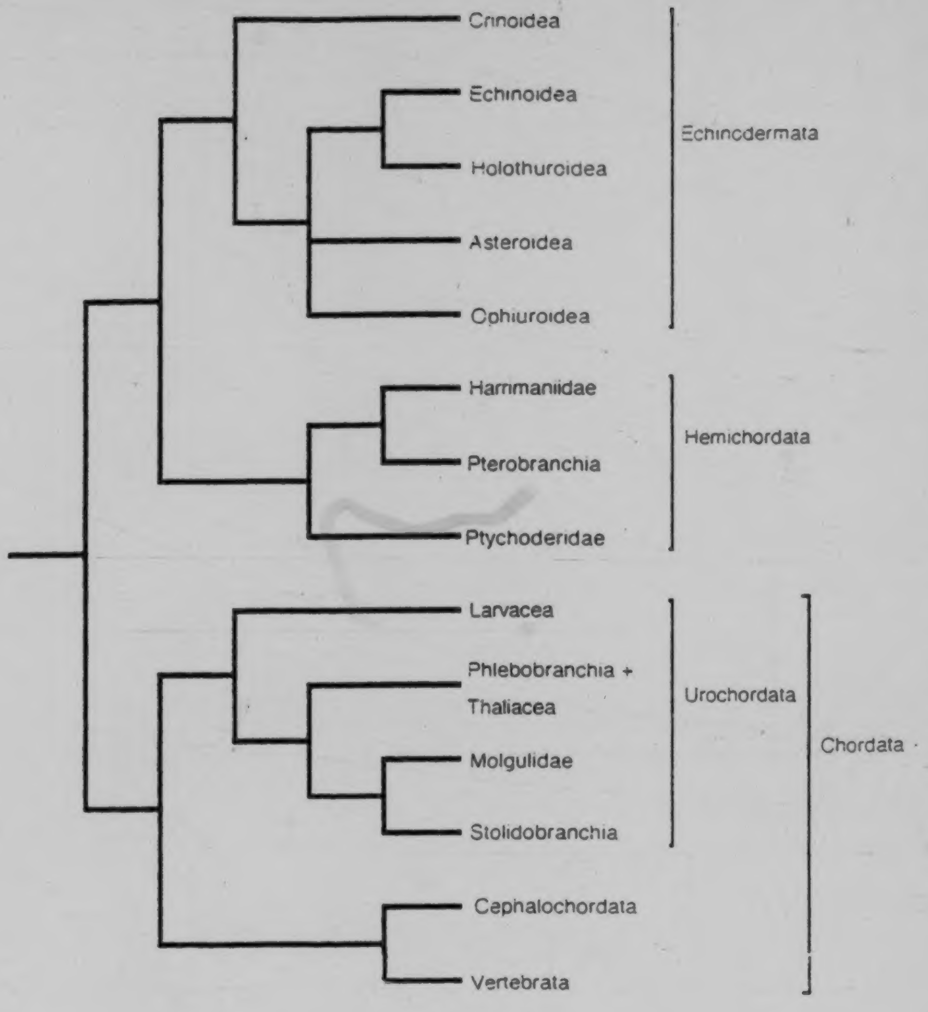
Crinoidea
Echinoidea
Holothuroidea
Asteroidea
Ophiuroidea
Echinodermata
Harrimaniidae
Pterobranchia
Ptychoderidae
Hemichordata
Larvacea
Phlebobranchia + Thaliacea
Molgulidae
Stolidobranchia
Urochordata
Cephalochordata
Vertebrata
Chordata

Figure III-2. Cladogram of deuterostome groups for which there is support from molecular and morphological data sets. Hemichordate family relationships are derived from the 18S rDNA analysis presented in this study. Echinoderm class relationships are derived from the morphological and molecular analysis of Littlewood et al (1997) (modified from Smith 1997). Urochordate relationships are derived from 18s rDNA data of Swalla et al. (2000). Chordate relationships are derived from Mallátt and Sullivan (1998). Relationships among phyla are from the present study.

Table III-1

18S rDNA SEQUENCES USED IN THIS STUDY

Species	Accession Number and Reference	Taxon
Phylum Hemichordata		
Class Enteropneusta		
Balanoglossus carnosus	D14359[7]	P1-ACORN
Ptychodera bahamensis	????????*	P2-ACORN
Saccoglossus sp. A	????????*	H1-ACORN
Saccoglossus kowalevskii	L28054[6]	H3-ACORN
Saccoglossus cambrensis	X59119[11]	H4-ACORN
Harrimania planktophilus	????????*	H5-ACORN
Class Pterobranchia		TUBE-DWELLING
Cephalodiscus gracilis	????????*	C-TUBE
Phylum Chordata		
Subphylum Vertebrata		
Homo sapiens	M10098[2]	HUMAN
Rattus norvegicus	K01593[1]	RAT
Petromyzon marinus	M97575[3]	HAGFISH
Subphylum Cephalochordata		
Branchiostoma floridae	M97571[3]	AMPHIOXUS
Subphylum Urochordata		
Order Larvacea		
Oikopleura species 1	D14360[7]	LARVACEAN
Oikopleura dioica	AB013014[12]	LARVACEAN
Oikopleura species 2	AB013015[12]	LARVACEAN
Order Thaliacea		
Thalia democratica	D14366[7]	SALP

Order Pyrosomida		
Pyrosoma atlanticum	AB013011[12]	PYROSOME
Order Doliolida		
Doliolum nationalis	AB013012[12]	DOLIOLID
Class Ascidiacea		
Order Enterogona		
suborder Phlebobranchiata		
Ascidia ceratodes	L12378[8]	A-ASCIDIAN
Ciona intestinalis	AB013017[12]	C-ASCIDIAN
Order Pleurogona		
suborder Stolidobranchiata		
Pyura mammillata	L12432[8]	Y-ASCIDIAN
Molgula oculta	L12432[8]	M-ASCIDIAN
Styela plicata	L12442[8]	S-ASCIDIAN
Herdmania momus	AF165827[13]	P-ASCIDIAN
Halocynthia roretzi	AB013016[7,12]	H-ASCIDIAN
Phylum Echinodermata		
Class Crinoidea		
Antedon serrata	D14357[7]	COMATULID
Class Asteroidea		
Asterias amurensis	D14358[7]	STARFISH
Class Echinoidea		
Strongylocentrotus purpuratus	L28056[6]	SEA URCHIN
Class Holothuroidea		
Stichopus japonicus	D14364[7]	SEA CUCUMBER
Class Ophiuroidea		
Ophioplocus japonicus	D14361[7]	BRITTLE STAR
Phylum Arthropoda		

Artemia salina	X01723[14]	BRINE SHRIMP
Tenebrio molitor	X07801[14]	MEALWORM
Phylum Annelida		
Nephtys hombergii	U50970[15]	POLYCHAETE

([1]Chan et al. 1984; [2]Torczynski et al.1985; [3]Stock & Whitt 1992; [4]Hedges et al. 1990; [5]Wada et al. 1992; [6]Turbeville et al.1994; [7]Wada & Satoh 1994; [8]Hadfield et al. 1995; [9]Halanych 1995; [10]Halanych 1996a; [11]Holland et al. 1991; [12]Wada 1998; [13]Swalla et al. 1999; [14]Nelles et al. 1984; [15]Nadot and Grant unpublished; *results from this study.)

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Chapter IV

The Anatomy, Life Habits and Later Development of a New Species of Enteropneust, *Harrimania planktophilus* (Hemichordata: Harrimaniidae) from Barkley Sound

Abstract. A new species of enteropneust *Harrimania planktophilus* lives intertidally and subtidally in mixed sediments in Barkley Sound, British Columbia, Canada. *H. planktophilus* has a long neck skeleton extending into the pharyngeal region. The collar (mesocoel) has complete dorsal and ventral mesenteries. The trunk (metacoel) has four distinct regions that can be recognized externally: the pharyngeal region, post-pharyngeal swelling, hepatic region and an undifferentiated intestine leading to the anus. The dorsal pharynx is large and has large gill slits without synapticles. Posterior to the gills is a saddle - like constriction followed by a short esophageal region and a long gonadal region. The dorsally paired gonads extend almost to the end of the trunk. Eggs in the ovaries appear amber yellow and the male gonads appear slightly paler. The trunk terminates at an anus with well developed sphincter muscles. *H. planktophilus* forms long sinuous burrows that are semi-permanent and shared. Females deposit a tubular egg mass in a burrow where the embryos develop. Gastrulation appears to be by invagination, followed by a ciliated stage which has a telotrochal swimming band, suggesting that the ancestor to *H. planktophilus* developed via a tornaria larva. Young juveniles emerge from the egg membrane with a post - anal tail and assume an interstitial burrowing life habit. The mode of development and presence of a post-anal tail suggests that *H. planktophilus* is closely related to *Saccoglossus*, and together they are related to the colonial Pterobranchia.

Introduction

The Enteropneusta is an enigmatic class of worms belonging to the phylum Hemichordata. Thirteen genera are currently recognized from four families; the Ptychoderidae including the familiar *Balanoglossus*, the monotypic hydrothermal vent Saxipendidae (Franzen 1985, Woodwick and Sensenbaugh 1985), the Spengelidae, and the Harrimaniidae including the genera *Protoglossus*, *Saccoglossus*, *Xenopleura*, *Stereobalanus* and *Harrimania*. About 20 species have been described from the family Harrimaniidae. Harrimaniids are the most morphologically simple of the Enteropneusta, they lack hepatic sacs, synapticles in the gill bars, and nerve roots extending from the collar cord. To date, developmental observations of the family Harrimaniidae have been restricted to *Saccoglossus* (Burdon-Jones 1952, Davis 1908, Colwin and Colwin 1953) and *Glandiceps* (Rao 1953). *Saccoglossus pusillus* (Davis 1908) is the only harrimaniid from the west coast of North America for which developmental information exists.

Ricketts et al. (1985), originally published in 1939, remains the most comprehensive review of the Enteropneusta from the Pacific coast of North America. The present status of the Enteropneusta from the Pacific coast of North America is briefly summarized here. From the family Harrimaniidae, *Saccoglossus* (formerly *Dolichoglossus*) *pusillus* (Ritter) (in Horst 1930, Davis 1908) is described from San Pedro and San Diego, California. In an earlier work Ritter (1902) indicates that this same animal is abundant in Puget Sound. Recently, the *Saccoglossus* from Puget Sound was described by King et al. (1994), who considered it the same as *S. bromophenolosus* (formerly identified as*S. kowalevskii*) from the northeast coast of the United States. King's assertions are made on the shared presence of 2,4-dibromophenol in the two animals, but given that this same allelochemical is found in *Protoglossus graveolens* (Giray and King 1997), and that no comparison or distinction is made with S. *pusillus* from California, the status of the *Saccoglossus* species from Washington state remains uncertain. Two other apparently undescribed species of *Saccoglossus* occur on the Pacific coast, one is common subtidally in Barkley Sound, British Columbia (Cameron and Mackie 1996, Cameron et al. 1999) and the other is from the Santa Maria Basin (Woodwick1996). Molecular tools will go a long way in revising the taxonomy of the genus from North America. *Stereobalanus* sp. has been discovered from the Santa Maria basin (Woodwick 1996) and *Harrimania maculosa* (Ritter 1900), is described from Alaska.

Two undescribed species of *Schizocardium*, from the family Spengelidae, have been found on the coast of California, the first from Mugu Lagoon (Ricketts et al. 1985) (now a military base) and in Newport Bay (Ricketts et al. 1985) (which is now a highly modified and heavily populated shoreline), and the second from the Santa Maria Basin (Woodwick 1996). From the family Ptychoderidae Ritter (1902) mentions *Balanoglossus occidentalis* from San Pedro, California. Another *Balanoglossus* has been collected from the San Pedro Basin (Woodwick 1996) and a large individual occurs on the northeast coastline of the sea of California (author's collection). *Glossobalanus berkeleyi* (Willey 1931), originally described from Nanaimo, B.C., is no longer present in Nanaimo Bay but is found in Puget Sound, WA (author's records). The Ptychoderidae is the morphologically most complex of the enteropneust families and typically develops via a tornaria larva. Two species of tornaria larvae have been described from western North America see Ritter and Davis (1904), but the corresponding adult worms are unknown.

Given the paucity of information on enteropneusts from the west coast of North America, the purpose of this study is to describe a new member of the Harrimaniidae, *Harrimania planktophilus* and to present observations of its life habits and development.

Materials and Methods

Harrimania planktophilus was collected from the intertidal sand flat at Cape Beale (48° 47' 30" N, 125° 12' 56" W) in Barkley Sound, Vancouver Island, Canada. Six worms were collected during February 1997 and another twenty one were collected over the summer of 1999 and transported back to the Bamfield Marine Station where they were kept in specimen bowls containing their natural sediment under a flow of fresh sea water. One or two worms at a time were removed from their bowls for embedding in paraffin wax for histology. Animals were allowed to defecate their gut contents and then cleaned externally of sediment before relaxing in 7% $MgCl_2$ followed by fixation in Bouin's solution and dehydration in a graded series of ethanol. Once in 100% ethanol, animals were dissected into small pieces, transferred to xylene, followed by infiltration of paraffin wax. Sections were cut on an American Optical Corporation "820" Spencer microtome and stained with either Delafield's hematoxylin or eriochrome - cyanin, and viewed and photographed with an Olympus OM-4T 35 mm camera on a Olympus BH2 compound microscope.

Blastula were obtained from a single egg mass in aquaria on February 19, 1997. Approximately 70 embryos were in the cylindrical embryo mass, 20 of which were removed and reared in finger bowls containing fresh sea water which was changed twice a day. High mortality was observed in these first 20 embryos, and so subsequently, only one or two embryos was removed from the egg mass at a time to reconstruct the development. The temperature of the sea water during the observation period varied between 10 and 11° C. The various stages were measured, photographed, described and drawn. I was unable to study unfertilized eggs, fertilization, and early cleavage stages preceding the late blastula stage.

Taxonomic treatment

Harrimania Ritter 1900

Harrimania Ritter 1900: 112.
TYPE SPECIES: *Harrimania maculosa* Ritter 1900: 112-115.

Harrimania planktophilus new species
Figs. 1, 2, 3.

Description. External

Harrimania planktophilus is, in general, muscular and active. Because it is robust, it can be collected completely intact, unlike many other species of enteropneust, which are fragile and break easily. *H. planktophilus* (Fig. 1A) is small with respect to other enteropneusts, the longest individual was 6 cm in length when extended in the bottom of a specimen bowl and the average specimen was approximately 3.5 cm. The proboscis is conical, longer than broad and about twice as long as the collar with a middorsal groove. The groove extends two-thirds the length of the proboscis from the posterior margin. In some animals the neck skeleton is pigmented black and can easily be seen through the neck epithelium. The collar is shorter than broad (Fig. 1B) with a distinctive circumpharyngeal groove about half way between anterior lip and posterior marginal ridge. The posterior ridge has a fine crease that accompanies it. Anteriorly the collar lip is muscular and contractile, altering the circumference and shape of the mouth. A posterior neuropore is always present and appears rust colored around its perimeter. The trunk can be separated into four regions; a long anterior pharyngeal region, a reddish coloured post-pharyngeal region, a short transparent region followed by the darkly pigmented digestive trunk which has no liver sacs and a posterior region with a terminal anus with a sphincter muscle. In gravid males and females the gonads are paired and located dorsolaterally from the posterior branchial region to the anus, in the form of large, irregular masses. The pharyngeal region has many (36.2 ± S.D. 9.7; N=6) pairs of large, muscular gill pores which open to the outside in paired dorso-lateral grooves. A strong current can be observed in animals acclimated to cold (5-7 ^{0}C) sea water, and when the pores are expanded the tongue bars can be seen in the pharynx. Approximately 1 in 10 animals has black skeletal bars, rather than having the more common collagenous opaque white color. The pharyngeal region is 5 times the length of the extended proboscis. The post-pharyngeal region has a large bilobed muscular organ that is pigmented dark red, and posterior to this the meandering gut can be seen through the body wall. The following measurements in millimeters are from an average adult living specimen; length of proboscis 2; length of collar 1; length of pharynx 9; length of post pharynx to anus 20; total 32.

Color. Cream colored proboscis (pl XVI, f-19), capucine orange (pl III, d-13) collar, pale yellow-orange trunk (pl III, f-15) except for the post pharyngeal organ which is brick red (pl XIII, k-5) and the hepatic region which is brownish-olive (pl XXX, m-19) (Ridgway 1912). The eggs appear amber yellow (pl XVI, b-21) and male gonads slightly paler.

Description. Anatomical and histological characters

Proboscis The epithelium of the proboscis is simple and columnar with multiciliated cells and glandular cells having basal nuclei. The nervous layer of the ectoderm is about equal in thickness to the underlying circular muscle layer, and thickening slightly under the dorsal proboscis groove. Longitudinal muscle fibers are arranged in radiating plates (Fig. 2A) as in *Ptychodera* and *Stereobalanus* (Woodwick 1996), less than 50, with space between and narrowing near the center where the proboscis coelom is small or completely absent. There is no ventral proboscis septum, instead a plate of connective tissue underlies the ventral stomochord and extends ventrally, wedging itself between two radial muscle plates, reaching nearly half way to the ectoderm. This connective tissue is contiguous with the third posterior portion of the proboscis stomochord (Fig. 2A inset) and becomes continuous with the neck skeleton posteriorly. The stomochord, which lacks the vermiform process of *Schizocardium* (Horst 1939), is composed of columnar cells arranged radially around a central ciliated lumen, each cell with a large vacuole distally and a proximal nucleus. The walls of the dorsal blood vessel form an extensive glomerulus to the left and right of the stomochord and are confluent over its anterior end. Pericardial sac is not well developed and forms a wedge, which extends dorsally from blood vessel between two radial muscle plates, and extending nearly half way to the epidermis.

The proboscis coelom is small to absent, is never divided completely, and extends posteriorly on the ventral and dorsal side of the stomochord. Ventrally the coelom ends in a blind sac, and dorsally it extends extremely posteriorly and opens to the outside through a single proboscis pore just left of the dorsal midline and almost into the anterior neuropore.

Collar The dorsal collar cord sometimes has and sometimes lacks an anterior neuropore, and always has a posterior neuropore. Over its length one or two small lacunae may occur laterally. Giant cells are absent. A dorsal crest is present throughout length of the collar cord (Fig. 2C). The perihaemal diverticula are well developed and extend two thirds the length of the collar; the septum between them is well defined. Anteriorly the diverticula become confluent, reduced in size, and enveloped in collagenous tissue that is extensive with the neck skeleton. The dorsal blood sinus is situated between the collar cord dorsally and perihaemal diverticula ventrally, and infrequently situated between septa. The collar coelom extends far into the neck. Peribuccal spaces are apparently absent. Crura of neck skeleton extend into the pharyngeal region. The body of the skeleton also extends far back, reaching beyond the middle of the collar before bifurcating. The notochord consists of two distinct parts, the anterior pouch - shaped part like that found in all Hemichordata, and a posterior gutter shaped part continuous with the pouch - shaped part, and extending into the anterior esophagus before ending in two blind tubes. It is similar in design to that of *Harrimania maculosa* (Ritter 1900), but not nearly so extensive.

Trunk Poorly developed longitudinal folds separate the dorsal pharyngeal trunk from the ventral digestive trunk (Fig. 2D). The gill slit skeletal bars are commonly opaquely colored, but in one in every ten animals are pigmented black. Peripharyngeal diverticula extend into the tongue bars only. No synapticles join the primary gill bars with the tongue bars. The atrial canal is u-shaped, heavily ciliated and leads from the pharyngeal slits to the outside via a ciliated pore. An epibranchial ridge is absent. *Harrimania planktophilus* is dioecious. Gonads are restricted to the posterior two thirds of the trunk perivisceral coelom. Gonadal pores occur dorsolaterally. Immediately behind the pharynx the enteric epithelium thickens laterally (Fig. 2E) forming two opposing lobes, called the post-pharyngeal organ, and the lumen is correspondingly reduced in size as compared to the abdomen farther back. The gut takes a meandering route to the anus, is lined by a simple epithelium, and hepatic sacs are absent. A ventral longitudinal muscle layer is broad and well developed and narrows dorsally (Fig. 2E). Circular muscle is absent in this part of the body. The subepidemal nerve layer is thickened on the dorsal midline and even more so on the ventral midlines (Fig. 2F) and therefore may be the main conductive channel in the trunk, similar to a sympatrically occurring and apparently undescribed *Saccoglossus* sp. (Cameron and Mackie 1996).

Diagnosis

The adult animal is distinguished by having a single proboscis pore, a glomerulus that extends frontally over the stomochord, a neck skeleton that extends into the pharyngeal trunk. A stomochord extends posteriorly into the frontal region of the collar in the form of paired furled tubes. The collar has a complete dorsal crest. Animals may have as many as 54 pairs of gill pores. Gonads overlap with only the most posterior few gill pores and eggs are pigmented yellow.

Development

Embryos were found in aquaria at early to mid-blastula stage and therefore information about gamete structure, egg maturation, events of fertilization, early cleavage stages and blastulation are lacking. The coeloblastula (Fig. 3A) was 75 um in diameter and heavily pigmented lemon yellow. It developed in a fertilization membrane 83 µm across that had a thin (1.5um) sticky jelly layer. The development of the animal was often obscured by debris and sand adhering to the jelly coat. Gastrulation appeared to proceed by invagination of the posterior (vegetal) pole resulting in a hemispherical shape that persisted for about 12 hours (Fig. 3B). The wide archenteron almost completely obliterated the blastocoel. The blastopore began to constrict, changing the shape of the gastrula into a sphere (Fig. 3C). Over a period of 24 hours the blastopore shrank, finally persisting as a tear-drop shape. There were no external cilia.

The development of cilia initiated rotation about the embryonic chorion (fluid) forty two hours after discovering the embryos (Fig. 3D). In addition to rotation the 'larvae' propelled in the apical direction. A dimple was all that remained of the closed blastopore. The spherical larvae were 72 um in diameter. A wide telotrochal band formed on the otherwise uniformly ciliated ball. There was no ciliated apical tuft. Elongation by the constriction of a mid-ventral groove was followed by hatching of a 95 um long benthic pre-juvenile stage (Fig. 3E). As with the rest of the development, hatching was asynchronous. Of twenty individuals extracted from the burrow egg mass, only a single animal reared in culture hatched. Of approximately 50 embryos left in the burrow, 14 were burrowing juveniles were removed from aquaria. The fate of the remaining eggs was uncertain, but predation was not ruled out. Developing embryos were extruded from burrow systems and interstitial sediments in the field. Often the jelly layer was shed shortly before hatching of from a totally transparent chorion.

Elongation and differentiation of the body into distinct proboscis, collar and trunk was complete by day eight for the remaining few animals in culture (Fig. 3F). Body segment lengths were 55.0 um, 18.5 um, and 75.0 um respectively for the proboscis, collar and trunk. The animal could locomote on a plastic dish one body length every two seconds. Forward locomotion was guided by a ciliated muscular proboscis. Posterior locomotion was equally efficient and accomplished by a reversal in ciliary beat. Ciliation was most apparent on the proboscis tip, the collar, and the end of the tail. The pre-oral ciliary groove developed later, after about twelve days. The posterior ventral tail became post-anal with the completion of the alimentary canal. The tail extended from the ventral trunk and was heavily ciliated and glandular. The tail could adhere to sediments, plastic and glass. Like the adults, the juveniles moved away from light.

The first pair of gill slits was large and perforated the anterior pharynx (Fig. 3G). The circular slits, lined with long cilia, lacked any sign of a skeleton. When viewing proximally to medially ciliary waves pass in a clockwise direction on the left side and counter-clockwise on the right side of the animals resulting in a symmetrical ciliary activity. At day 17 the juveniles had a second pair of gill pores, without any skeleton and a long ventral post-anal tail (Fig. 3I).

Biology

Harrimania planktophilus is an active infaunal burrower. Individuals were found in two locations in Barkley Sound; subtidally in the Ross Islets (49° 52'N, 125° 10'W), and in the low intertidal of a protected beach adjacent to the eastern slope of the Cape Beale extrusion Beale (48° 47' 30" N, 125° 12' 56" W). At Cape Beale, *H. planktophilus* was collected at extreme low tides that occur during the morning hours in spring and summer and during the evening in fall and winter (Canadian Tidal Tables). *H. planktophilus* was typically found in a mixture of calcium carbonate biogenic debris and sorted sands with a low concentration of organics. The carbonate fraction of the Cape Beale deposits in the lower tidal range typically contained over 30% barnacle plates together with fragments of gastropods, and less abundant echinoids, bryozoa and foraminifera. The fragments were derived from invertebrates inhabiting the rocky shoreline surrounding the beach, and from input from outside the confines of the protected beach. The long - shore input of biogenic debris was indicated by scattered bivalve shell debris (bivalves were conspicuously absent from the immediate environment) and because much of the skeletal remains were worn, discolored, often heavily bored, and in some cases associated with relict gravel, suggesting the carbonates too, may have been relict.

H. planktophilus created sinuous interconnected tunnel systems that did not seem to approach the epibenthos. They were strongly photonegative, and did not smell of bromophenols. In aquaria, more than one animal would frequent a single burrow system. Embryos were deposited in the burrow, the egg mass was cylindrical in shape with a hollow center, through which adult worms and water current may pass. Embryos stuck to each other with a thin sticky jelly coat. The face of the embryo outer membrane directed into the cavity of the cylinder was clean and the face of the egg membrane directed towards the burrow wall was coated with sediment and detritus.

H. planktophilus ingest sediment that they trap on their proboscis with mucus (Fig. 4), and transport back to the mouth with cilia. They also suspension feed on interstitial plankton by propelling water into the mouth and out of the gill pores using pharyngeal cilia (chapter VII). Although debris acquired from pore water may comprise a small amount of the total gut content, it may have a significant role nutritionally.

Etymology

The species name is the Latin and means "plankton loving" reflecting the animals ability to suspension feed.

Holotype

Adult female, British Columbia, Vancouver Island, Barkley Sound, Cape Beale protected beach, north side adjacent to the "gap", 48° 47' 30" N, 125° 12' 56" W, 20 September 1999, C.B. Cameron.

Paratypes

None at present

Evidence for monophyly of the genus *Harrimania*

There are three families of Enteropneusta, four if one considers the deep sea hydrothermal vent enteropneust, *Saxipendium coronatum* (Woodwick and Sensenbaugh 1985), in a family of its own. *Harrimania planktophilus* belongs to the family Harrimaniidae, of which there are about 20 described species from the genera *Saccoglossus*, *Protoglossus*, *Stereobalanus*, *Xenopleura* and *Harrimania*. Harrimaniids have no liver-sacs, no synapticles joining the primary and secondary gill bars, and no nerve roots in the collar cord mesentery. *Harrimania* is distinguished from *Saccoglossus* by having a short proboscis and from *Xenopleura* by having large branchial pores. *Harrimania* is distinguished from *Protoglossus* by having a more developed gill skeleton, and by having a single atrium for each gill pore (in *Protoglossus* all of the pharyngeal pores are fused to form two parallel grooves into the branchial pharynx). *Harrimania* is distinguished from *Stereobalanus* by lacking a pair of genital wings.

Two other species of *Harrimania* are known: *H. maculosa* (Ritter 1900) and *H. kupfferi* (Spengel 1901, in Horst 1939), both of which have, like *Stereobalanus*, paired proboscis pores. *H. maculosa* is a common intertidal form that seems to be a feature of under - rock collecting at Kodiak, Prince William Sound, Orca and Valdez. This thick, dark-brown acorn worm is about 12 cm long and emits a strong bromophenol odor (Ritter 1900). It does not burrow as do most enteropneusts, but lies under stones after the fashion of some holothurians. In all of these respects *H. maculosa* is unlike its British Columbia cousin, *H. planktophilus*. Perhaps the most conspicuous feature of *H. maculosa* is it's extensive esophageal stomochord. *H. maculosa* always has two proboscis pores, and has an epibranchial ridge.

H. kupfferi is from Kattegatt straight in Scandenavia, Oresund and Hellebaek, East of Laesso Island and Greenland (Horst 1939). *H. kupfferi* is 8 - 9 cm long, with a pale colored proboscis and collar, reddish gonads and a brown trunk. It differs from *H. planktophilus* in that its glomerulus halves do not connect frontally. The legs of the neck skeleton are not as extensive, extending to the posterior end of the collar, whereas in *H. maculosa* and *H. planktophilus* they extend into the trunk. *H. kupfferi* has no mesentery separating the coelomic cavities in the dorsal and ventral collar. The ventral pharynx is larger than dorsal pharynx, up to 40 gill pores (Horst 1930), whereas in *H. planktophilus* has a larger dorsal pharynx and as many as 54 pairs of gill pores.

Phylogenetic relationships

The phylogenetic position of *Harrimania* within the family Harrimaniidae is not precisely known. However, morphological data suggests that the genus is more closely related to *Stereobalanus* than to any other genus. DNA - sequence data (Cameron et al., unpublished) indicate that *Harrimania* is more closely related to *Saccoglossus*, than *Ptychodera*, *Balanoglossus*, *Rhabdopleura* or *Cephalodiscus*. These are the only genera for which DNA data presently exist.

Keys to the families and genera of the Enteropneusta, and to the species of the genus *Harrimania*

A. Notochord with a vermiform process; pericardium with
anterior diverticula more or less developed .... **Spengelidae**

(a) Liver-sacs and synapticles present; gill-slits almost
equalling the pharynx in depth, so that the ventral,
non-branchial part of the pharynx is reduced to a
mere groove; nerve-roots absent; pericardial diverticula long...... *Schizocardium*, Spengel (1893)

(b) Liver-sacs absent; ventral part of pharynx well developed;
pericardial diverticula short

(i) Synapticles and nerve-roots absent,

(*a*) Peribuccal spaces... *Willeyia*, Punnett (1903)

(β) Without peribuccal spaces.................................... *Glandiceps*, Spengel (1893)

(ii) Synapticles present; nerve-roots present or absent;
genital region with dermal pits... *Spengelia*, Willey (1898)

B. Notochord with no vermiform process; pericardium simple;
ventral part of pharynx large, and sometimes more or less
separated from the branchial part.

(a) Liver-sacs, synapticles and nerve-roots present.................. **Ptychoderidae**

(i) Genital wings well developed.

(*a*) Gill-sacs opening by long slits.................................. *Ptychodera*, Eschscholtz (1825) (β) Gill-sacs opening by small pores............................ *Ballanoglossus*, Delle Chiaje (1829)

(ii) Genital wings hardly developed.................................. *Glossobalanus*, Spengel (1893)

(b) Liver-sacs, synapticles and nerve-roots absent.................... **Harrimaniidae**

(i) With many atria

(*a*) Proboscis long; one proboscis pore;

(1) burrowing.. *Saccoglossus*, Spengel (1893)

(2) non burrowing.. *Saxipendium*, Woodwick (1985)

(β) Proboscis short;

(1) one proboscis pore; viviparous........................ *Xenopleura*, Gilchrist (1925)

(2) usually two proboscis-pores.

(a) Two pairs of genital wings;.......................... *Stereobalanus*, Spengel (1901)

(b) No genital wings; .... *Harrimania*, Ritter (1900)

(I) Neck skeleton extending into trunk,
with collar mesenteries

- Extensive branchial ridge and
collar stomochord.............................. *H. maculosa*, Ritter

- Reduced branchial ridge and
reduced collar stomochord.................. *H. planktophilus*, Cameron

(II) Neck skeleton not extending
into trunk, without collar mesenteries.......... *H. kupfferi*, Spengel

(ii) Without atria... *Protoglossus*, Caullery & Mensil (1904)

Figure IV-1. A. *Harrimania planktophilus*. Total length of relaxed and uncoiled animal is approximately 32 mm.

Figure IV-1. B. Light micrograph of a longitudinal section of *Harrimania planktophilus*. bs, branchial sac (= atrium); ep, epithelium; g, glomerulus; gb1, primary gill bar; gb2 secondary (or tongue) gill bar; gl, gut lumen; lm, longitudinal muscles; mc, mesocoel; mo, mouth; ov, ovaries; pr, proboscis; ps, proboscis skeleton; st, stomochord; tc, trunk coelom (or metacoel); vc, ventral cord.

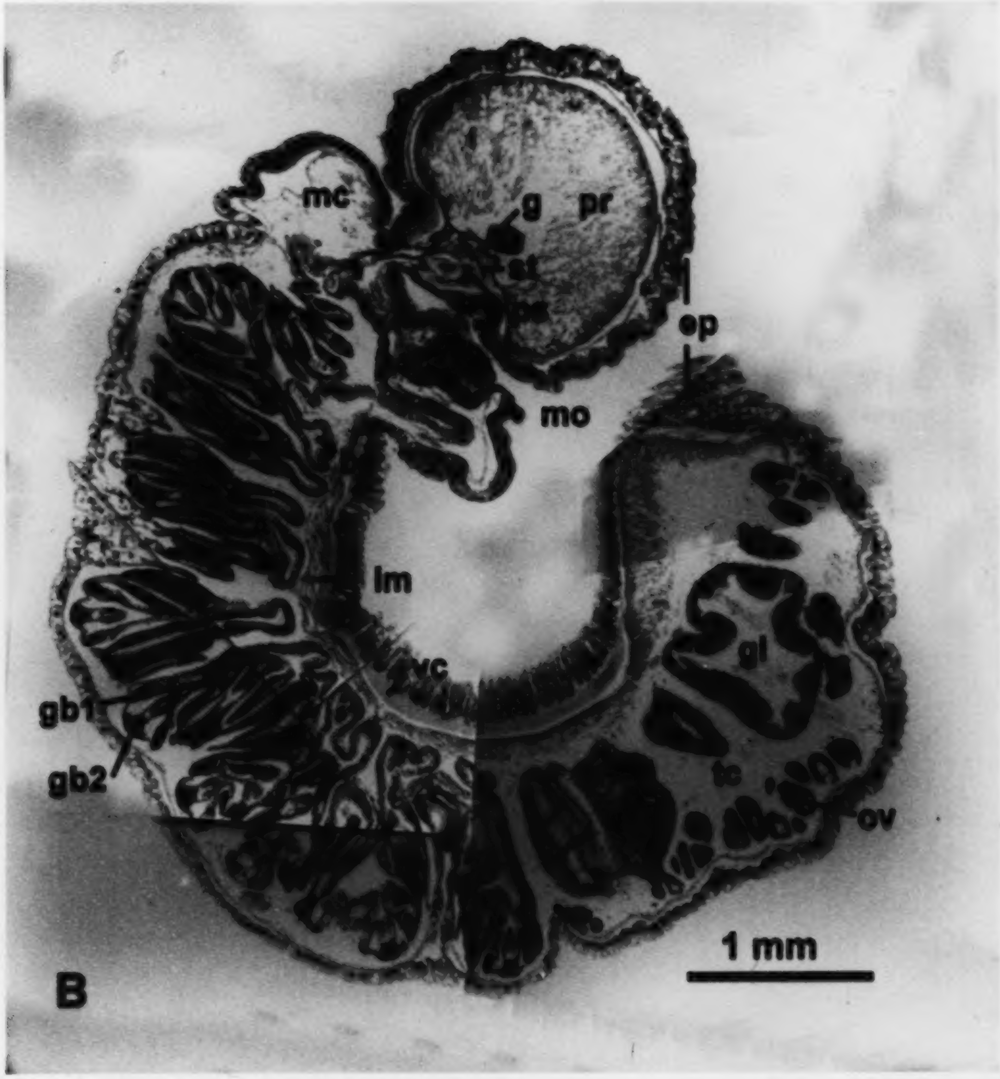
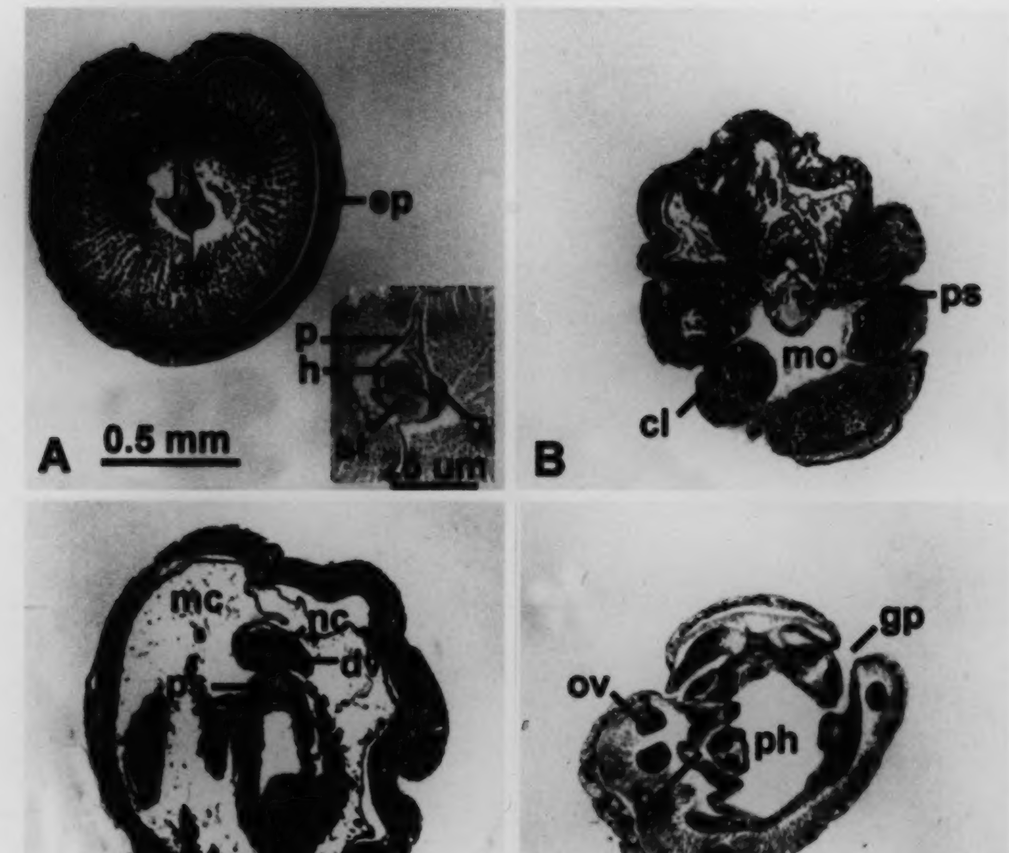

mc
g
pr
st
ep
mo
lm
vc
gb1
gb2
gl
tc
ov
1 mm
B

Figure IV-2. *Harrimania planktophilus.* Light micrographs of transverse sections. A. Proboscis. Inset, heart - kidney coelomic complex. B. Neck and anterior collar lip. C. Collar. D. Pharyngeal trunk. E. Post - pharyngeal organ. F. Abdomen. bs, branchial sac (= atrium); cl, collar lip; dv, dorsal vessel; ep, epithelium; g, glomerulus; gl, gut lumen; gp, gill pore; h, heart; lm, longitudinal muscles; mc, mesocoel; mo, mouth; nc, nerve cord; ov, ovaries; p, pericarduim; pc, proboscis coelom (or protocoel); ph, pharynx; ppo, post - pharyngeal organ; ps, proboscis skeleton; st, stomochord; tc, trunk coelom (or metacoel); vc, ventral cord.


op
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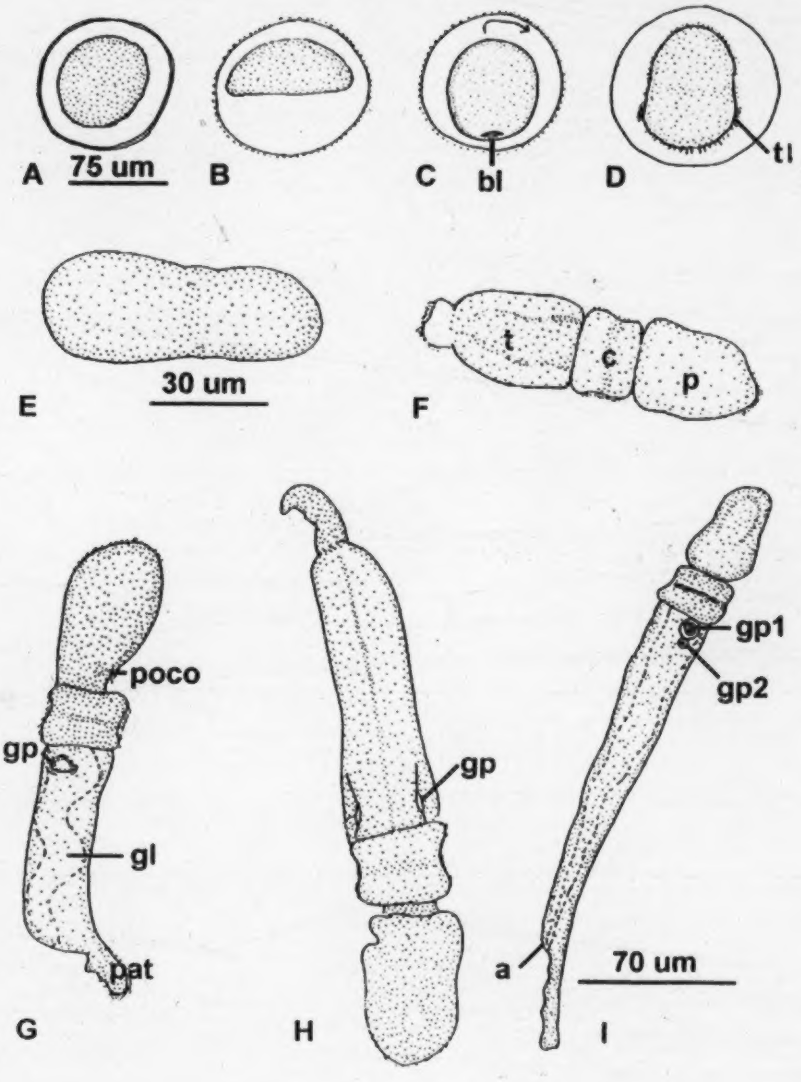
F

Figure IV-3. Schematic drawings of the later development of *Harrimania planktophilus*. A. Blastula. B. Gastrulation showing obliteration of blastocoel. C. Gastrula; arrow indicates direction of rotation. D. Ciliated ball with vestigial telotroch. E. Newly hatched juvenile. F. Juvenile with distinct body regions. G. Juvenile at first gill pore stage, lateral view. H. Juvenile at first gill pore stage, dorsal view. I. Juvenile at second gill pore stage, lateral view. a, anus; bl, blastopore; c, collar; gl, gut lumen; gp, gill pore; gp1, first gill pore; gp2, second gill pore; p, proboscis; pat, post - anal tail; poco, pre - oral ciliary organ; t, trunk; tl, telotroch.


A
75 um
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a
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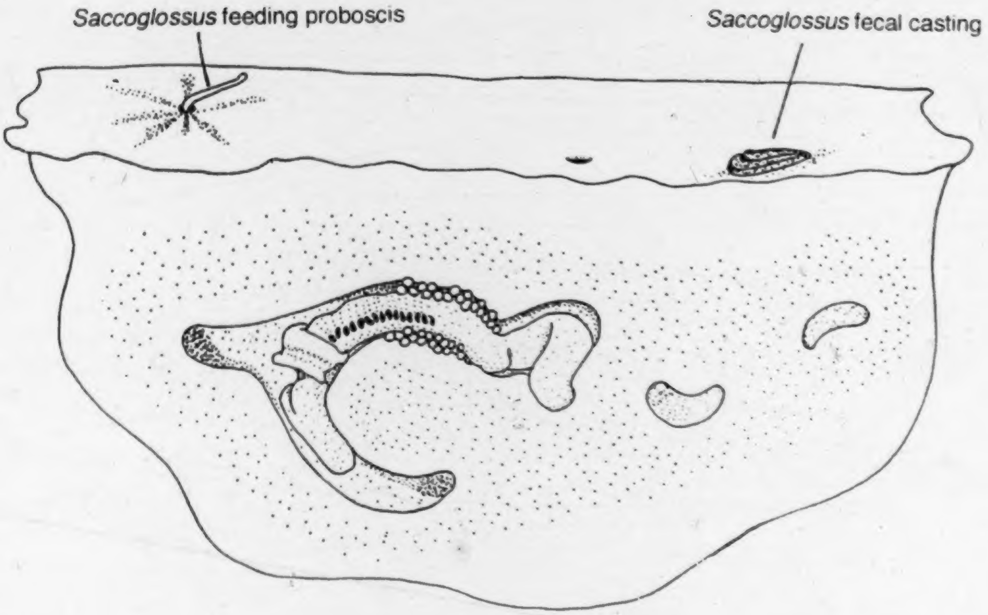
Figure IV-4. Schematic drawing of *Harrimania planktophilus* in situ with cylindrical shaped mass of embryos.


Saccoglossus feeding proboscis
Saccoglossus fecal casting

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Chapter V

Conduction pathways in the nervous system of *Saccoglossus* sp. (Enteropneusta)*

Abstract A species of *Saccoglossus* from Barkley Sound, British Columbia, was observed in the field and found to exhibit a startle withdrawal response. Optical and electron microscopy of the nerve cords failed to reveal giant axons. The dorsal collar cord and ventral trunk cord consist of small axons with a mean diameter of 0.4 μm. The majority of the axons run longitudinally and there is no indication of a specialized integrative center. Electrical recordings from the nerve cords show events interpreted as compound action potentials. The potentials are through-conducted from proboscis to trunk. Such propagated events probably mediate startle withdrawal. Conduction velocities did not exceed 40 cm • s^{-1} in any part of the nervous system.

*A version of this chapter has been published as: **Cameron, C.B. and G.O. Mackie. 1996.** Conduction pathways in the nervous system of *Saccoglossus* sp. (Enteropneusta). Canadian Journal of Zoology 74:15-19.

Introduction

The enteropneusts, with the pterobranchs, constitute the small deuterostome phylum Hemichordata, generally regarded as an early offshoot from the chordate line of evolution (Ruppert and Barnes 1994; Wada and Satoh 1994). Such a group might be expected to hold clues concerning early chordate neural evolution, but the nervous system and behaviour have been little studied since the work of Bullock (1940; 1944; 1945). The nervous system appears to be very primitive, consisting of an intraepithelial nerve plexus thickened locally into longitudinal fiber bundles or "cords". In the collar region the dorsal cord sinks below the surface in a manner reminiscent of the formation of the dorsal neural tube in vertebrate embryos. Despite its internal location, the collar cord does not resemble an integrative center histologically (Bullock 1945; Bullock and Horridge 1965; Silen 1950; Knight-Jones 1952; Dilly et al. 1970). It appears to be a transmission pathway much like the other nerve cords, but with the interesting addition of giant axons.

Giant axons have been found in several species of enteropneusts (Spengel 1893; Bullock 1944). Their cell bodies are located in the collar cord and their axons decussate and run back into the general epithelial plexus of the trunk or into the ventral cord, presumably terminating in the longitudinal muscles. Their numbers are variable: Bullock (1944) counted between 15 and 20 in *Glossobalanus minutus*, fewer than a dozen in another species of *Glossobalanus*, and 161 in a *Balanoglossus* species. Counts for species of *Saccoglossus* range from 15 to 30. One electron microscope study (Dilly et al. 1970) has confirmed their presence in a member of this genus.

Most of what is known of enteropneust behavioural physiology is summarized by Bullock and Horridge (1965). Conduction is diffuse and decremental in many regions but through-conduction pathways are also thought to exist. The clearest example of a through-conducted response is the startle withdrawal response (Bullock 1940), where a gentle poke on the proboscis results in a rapid contraction of the longitudinal muscles of the trunk. Observed in the dark, startle withdrawal is accompanied by luminescence (Baxter and Pickens 1964).

The only neurophysiological investigation of an enteropneust reported to date is that of Pickens (1970) on *Ptychodera* sp. In this study, through-conducted signals were recorded from the dorsal and ventral trunk nerve cords and from the collar cord. Pulse amplitude was found to vary with shock strength. This, together with other evidence, suggested that the signals were compound action potentials. No fast pathways suggestive of giant axons were discovered and an electron microscope examination showed that most fibers in *Ptychodera* sp. were less than 0.33 µm in diameter (Pickens and Ferris 1969).

Given the paucity of information on enteropneust neurophysiology, neural fine structure, and behaviour, we decided to investigate a species of *Saccoglossus* from Barkley Sound, British Columbia. Our goals were (*i*) to determine if this enteropneust showed a startle response, (*ii*) to carry out a microscopical examination of the nerve cords with a view to describing the numbers and distribution of giant axons, which we assumed would be present, as in other members of the genus, and (*iii*) to record electrical events from its nerve cords to determine if through-conduction pathways existed as reported by Pickens (1970) for *Ptychodera* sp., but with the additional expectation of finding fast pathways corresponding to the giant axons.

Materials and Methods

The species of *Saccoglossus* used for this study has not yet received a formal taxonomic description, and will be provisionally designated *Saccoglossus* species A, pending determination by a specialist. This species is quite distinct from the only other enteropneust known to occur locally, *Saccoglossus bromophenolosus* (King et al. 1994). The two species differ in size, coloration, and habitat. *Saccoglossus bromophenolosus*, from Padilla Bay and Willapa Bay, Washington (Woodwick 1951), is an intertidal mud dweller, while species A has most frequently been found subtidally in coarse-grained calcereous debris of biogenic origin. Specimens were obtained at approximately 10 m depth in the Ross Islets, Barkley Sound, during the summer and fall, 1994. Field observations were made by SCUBA on the enteropneusts in their natural habitat and in aquaria at the Bamfield Marine Station. Specimens were transported to the University of

Washington Laboratories at Friday Harbor, where the electrophysiological recordings were made, and to the University of Victoria, where the optical and electron microscope work was carried out.

Specimens were fixed in Bouin's fixative for paraffin embedding. Four specimens were serially sectioned from proboscis to trunk. A single animal was fixed in 2.5% glutaraldehyde in 0.2 M Millonig's phosphate buffer at pH 7.4 followed by postfixation in 1% osmium tetroxide in 0.2 M phosphate buffer. Pieces of tissue were dehydrated and embedded in Epon 812. Thick epon sections (ca. 1.0 µm) were cut at about 180 points along the length of body from posterior proboscis to anterior trunk and stained in Toluine Blue. Thin sections were cut from the same blocks and were stained with uranyl acetate and lead citrate for electron microscopy.

Twenty specimens were used for recordings of nervous activity. They were pinned out on Sylgard platforms. Long thin polyethylene suction electrodes with internal tip diameters of 50 – 100 µm were used. Flexibility was important because the worms showed a great deal of peristaltic movement, tending to dislodge the electrodes. Recordings from the collar cord were made by removing the overlying tissues, exposing the cord. Signals were amplified, digitized, and displayed on an oscilloscope using conventional procedures. Stimuli were delivered through bipolar metal electrodes held in a micromanipulator. Shocks of 2 ms duration in the 5.0- to 10.0-V range were usually effective.

Results

Histology and ultrastructure

Sections were cut through the nerve cords in the proboscis, collar, and trunk. Particular attention was paid to the collar and anterior trunk region, where giant axons have been described in other species of *Saccoglossus* (Bullock 1945). Sections were cut through the collar cord (Fig. 1A) and selected regions were examined by electron microscopy (Fig. 1B). Most of the cord tissue consists of longitudinal bundles of small axons seen as subcircular profiles in sections cut transversely to the body axis. There is no suggestion of a cortical region with cell bodies surrounding a central neuropil. The axons are unsheathed and contain microtubules, mitochondria, and dense-cored and clear vesicles, as described by previous workers (Pickens and Ferris 1969; Dilly et al. 1970). In the collar cord and ventral trunk cord, axon diameters were found to vary within the range 0.1 – 1.3 μm, showing a normal distribution, with a mean of around 0.4 μm (Fig. 2). No giant axons were found in any part of the nervous system examined.

Behavioural observations

Specimens observed in the natural habitat showed a "startle" response, pulling themselves rapidly down into their burrows, as described in *S. pusillus* by Bullock (1940). In aquaria, the response was evoked by tactile stimulation and by gently tapping the wall of the tank. In the field, the approach of a SCUBA diver would initially evoke slow withdrawal, but any sudden movements in the water near the animal or disturbance of the sediment layer within approximately 1 m of its burrow evoked the startle response. The animals are evidently sensitive to vibrations transmitted through both the water and through the substrate. Withdrawal can occur when the proboscis and collar are extended above the substrate and seems to be dependant on the corkscrew-like orientation of the animal in the substrate and the contraction of the longitudinal muscles of the trunk (C.B. Cameron and A.R. Fontaine, unpublished data). These muscles are most strongly developed medioventrally.

Electrophysiology

Single electric shocks applied to the longitudinal cords in several regions evoked trains of propagated electrical events (Figs. 3a, 3b). These events rarely exceeded 50 μV in amplitude and were often so small as to be indistinguishable from base-line noise. The best recordings were obtained from the neck of the proboscis, where numerous nerve bundles converge and enter the collar, and from the ventral cord of the trunk. Signals were also recorded from the collar cord, but we were unable to record signals consistently from the dorsal trunk cord or the lateral body wall. Increasing the strength of shocks above the threshold for production of propagated events typically increased their amplitude and complexity, indicating that they are compound action potentials. Bursts of potentials evoked by single shocks often lasted for more than 50 ms, typically showing declining amplitudes. The earlier (more rapidly propagated) events in such series sometimes showed fairly consistent wave forms following shocks of similar strength given a few seconds apart (as in Fig. 3b), but we never saw potentials of the sort generated by giant axons in other animals, i.e., large, sharp, spikey events conducted at conspicuously high velocities, whose amplitudes are not affected by variations in shock strength.

It was possible to demonstrate through-conduction following single shocks within the dorsal proboscis cord, the collar cord, and the ventral trunk cord. The highest velocities were seen in the anterior region of the ventral trunk cord, regarded as the major nerve pathway in the trunk (Bullock 1945). Conduction velocity declined posteriorly. Shocks on the neck of the proboscis and on the collar cord evoked potentials that propagated through to the ventral cord, showing that there are continuous conduction pathways linking the proboscis with the trunk via the collar. These findings are summarized in Fig. 4 and Table 1.

These experiments were carried out on pinned specimens, so it was not possible to be certain that the trunk contractions exhibited following shocks in the anterior regions represent the startle withdrawals seen in the natural environment, but it is reasonable to assume that they do. The contractions are powerful and are through-conducted, occur with short latency rather than spreading by peristalsis, and primarily involve the longitudinal muscles of the anterior trunk region.

Discussion

The results reported here show that *Saccoglossus* sp. A has a startle response similar to that described in other enteropneusts, and demonstrate the existence of through-conduction pathways within the proboscis, collar, and trunk. These pathways are located in the dorsal nerve cords of the proboscis and collar and in the ventral cord of the trunk. They probably mediate the startle response, but a fuller electrophysiological analysis on unrestrained animals would be necessary to demonstrate this conclusively.

Knight-Jones (1952) showed that propagation of contraction waves in the trunk during fast withdrawals was blocked by lesions through the ventral nerve cord, indicating that the fast conduction pathways were located in this part of the nervous system. The fastest pathways identified in the present study were also in the ventral cord. The ventral cord lies directly adjacent to the trunk muscles whose contraction brings about the startle withdrawal.

The electrical potentials recorded in this study closely resemble those published by Pickens (1970) for *Ptychodera* sp. In neither case was there any indication of fast pathways of the kind elsewhere associated with giant axons. The structural evidence reported here likewise indicates that giant axons are completely absent from the nerve cords. Instead, we seem to be dealing with conduction in bundles of small axons. A normal distribution of axon diameters is seen and none exceed 1.3 μm in diameter. Such units are evidently sufficient for through-conduction and for mediation of startle behaviour. The question of the function of giant axons in those species having them thus remains to be addressed.

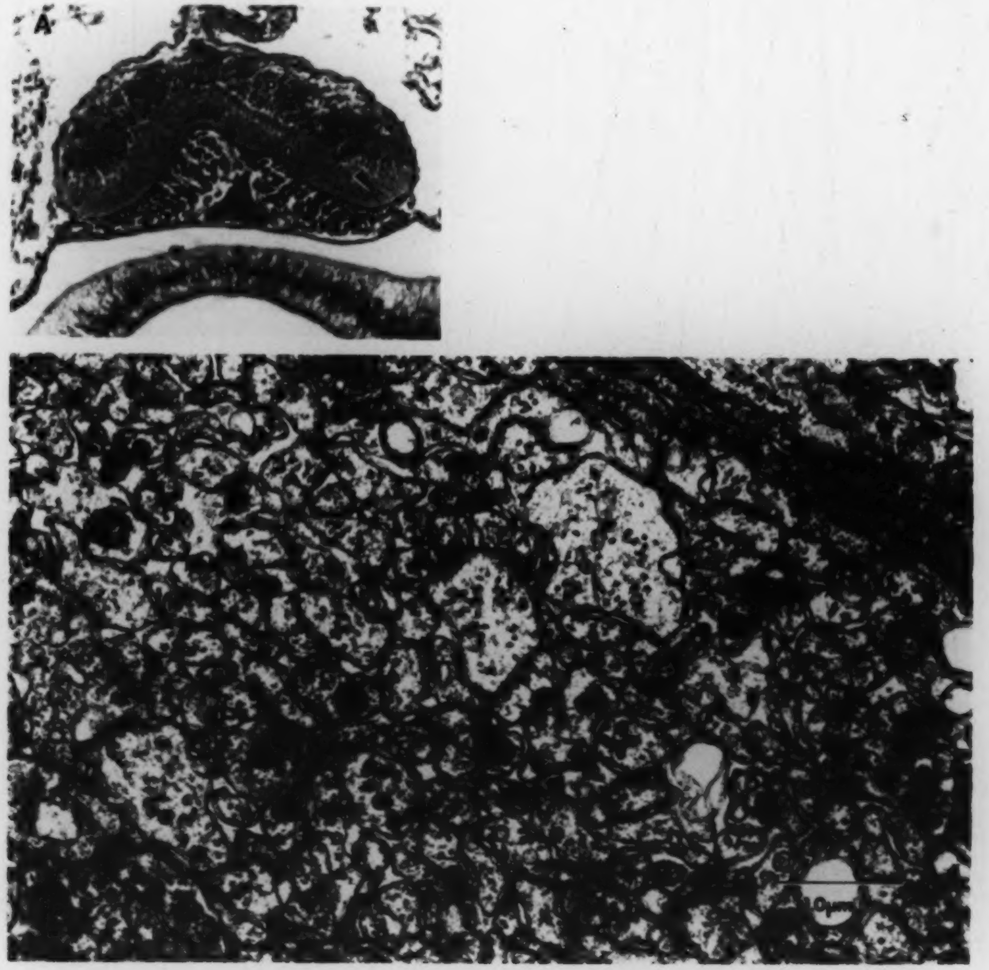
Our findings agree with those of previous workers who found no hint of "central" neural specialization in the dorsal cord. This structure appears to be a simple longitudinal transmission pathway linking the proboscis with the trunk rather than being specialized as an integrative center.

Recent insights into the patterns of expression of key developmental genes in insects and vertebrates have led to a revival of the old idea that somewhere in the line of chordate evolution, the dorsal and ventral sides became inverted (Arendt and Nübler-Jung1994). According to this view, the ventral nerve cord of annelids and arthropods is homologous to the dorsal nerve cord of chordates. How do enteropneusts fit into this picture? Commenting on the inversion theory, Peterson (1995) states that "many authors have accepted the homology between the dorsal nerve cords of enteropneusts and chordates, with both structures dorsal, hollow, possessing giant nerve cells, and formed by invagination or delamination of the neuroectoderm. The ventral nervous system of enteropneusts is clearly of invertebrate design with circumenteric connectives and a main ventral nerve cord." As Peterson points out, if structures homologous to both ventral and dorsal nerve cords exist in the same animal, it becomes impossible to argue that one represents the other.

To the present writers it is not obvious that the nerve cords of enteropneusts are homologous to either invertebrate ventral nerve cords or chordate dorsal nerve cords. The nerve cords of enteropneusts are local thickenings of the ectodermal nerve plexus; they show no special concentrations of nerve cell bodies and no ganglionic organization, and give off no nerves laterally. They remain intraepithelial even where, as in the collar, the epithelium containing the nerves becomes internalized. Though "hollow" in some species, this structure is quite unlike the dorsal tubular nerve cord of chordates and, as we have shown here, giant axons may be absent. It is true that "circumenteric connectives" exist in the form of tracts running from the ventral cord up and around the body wall on each side, converging toward the dorsal midline. There is also a nerve ring around the proboscis base. Such connectives would presumably be necessary in any worm-like animal possessing concentrations of nervous tissue on both dorsal and ventral sides, and it is by no means certain that they are the homologues of the circumenteric connectives of annelids and arthropods. Regarding the ventral nerve cord, this structure may be the "main" nerve cord in terms of the number of axons in it and their conduction velocity, but it is built on exactly the same principle as the other nerve cords, differing from them only in degree.

Rather than looking for homologies between the nerve cords of enteropneusts and those of arthropods and chordates, it seems to us more appropriate to regard the cords as ad hoc specializations of a diffuse ectodermal nerve plexus inherited from a common ancestor with the echinoderms. The equivalent system in echinoderms would be the ectoneural nervous system.

Figure V-1. Transverse sections through the collar cord. (A) Paraffin section through the collar cord, showing the nerve fibre layer (*nf*) beneath the epidermis (*ep*). (B) Electron micrograph of a section through the fibre layer in a region corresponding to that indicated by the arrowhead in A. Other parts of the fibre layer showed a similar range of axon diameters, and data from numerous sections such as this were used to prepare the histogram shown in Fig. 2.


A
1.0µm

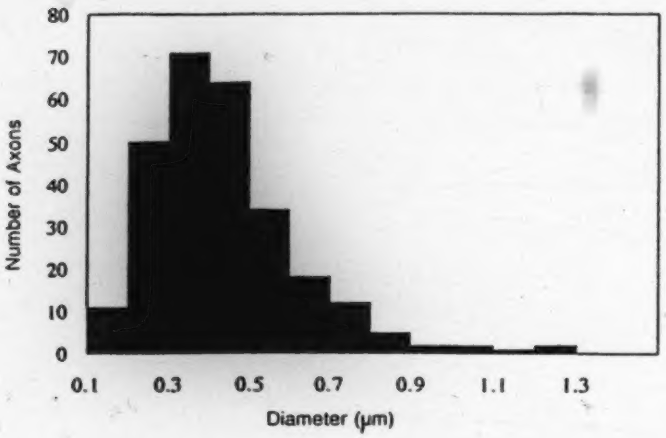


Figure V-2. Size-frequency histogram of axon diameters measured in the collar cord and ventral trunk cord: $n = 272$, $x = 0.438$, SD = 0.188.

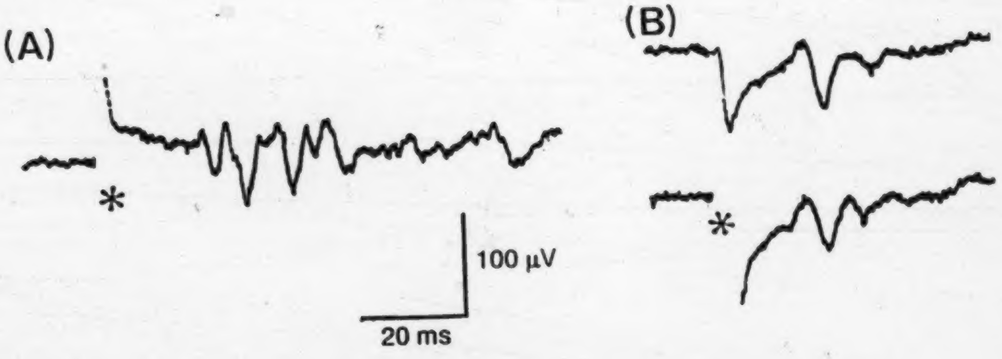


Figure V-3. Shock-evolved compound action potentials recorded extracellularly between two points on the dorsal midline of the proboscis (A) and ventral midline of the trunk (B, two sweeps, 1 s apart). Asterisks show shock artefacts. Conduction times were measured between the beginning of the shock artefact and the peak of the first, negative-going (downward) potential.

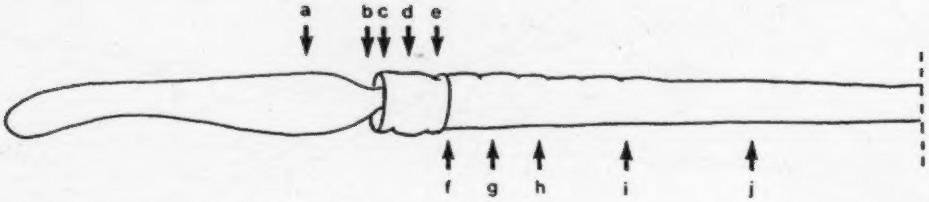


Figure V-4. Conduction velocities measured between various points along the dorsal and ventral midlines of *Saccoglossus* sp. Points *a* and *b* are on the proboscis, *c-e* are on the collar, and *f-j* are on the trunk (for details of paths see Table 1).

Table V-1. Lengths and velocities of conduction pathways.

Path	Path length (mm)	Conduction velocity ($cm \cdot s^{-1}$)
a–b	4.0	21.1
c–e	4.4	16.0
f–h	6.0	39.6
f–i	11.8	25.6
f–j	20.0	17.9
d–g	6.7	24.0
b–g	10.0	13.7

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Chapter VI

Gonadotropin-releasing hormone in mulberry cells of *Saccoglossus* and *Ptychodera* (Hemichordata: Enteropneusta)*

Abstract Mulberry cells are epidermal gland cells bearing a long basal process resembling a neurite and are tentatively regarded as neurosecretory cells. They occur scattered through the ectoderm of the proboscis, collar and anterior trunk regions of the acorn worms *Saccoglossus*, usually in association with concentrations of nervous tissue. They contain secretion granules that appear from electron micrographs to be released to the exterior. The granules are immunoreactive with antisera raised against mammalian and salmon gonadotropin-releasing hormone (GnRH). Similar results were obtained with another enteropneust, *Ptychodera bahamensis*, using antisera raised against tunicate-1 and mammalian GnRH. Mulberry cells were not found in either *Cephalodiscus* or *Rhabdopleura* (Hemichordata: Pterobranchia).

Extracts of tissues from 4200 *Saccoglossus* contain an area of immunoreactive GnRH that is detected by an antiserum raised against lamprey GnRH when characterized by high performance liquid chromatography and radioimmunoassay.

This is the first report of the occurrence of GnRH in hemichordates, probably the most primitive group clearly belonging to the chordate lineage. The physiological function of GnRH in enteropneusts is unknown, but an exocrine function appears more likely than an endocrine or neurotransmitter role.

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*A version of this chapter has been published as: **Cameron, C.B., Mackie, G.O., Powell, J.F.F. and Sherwood, N.M. 1999.** Gonadotropin-releasing hormone in mulberry cells of *Saccoglossus* (Hemichordata: Enteropneusta). Gen. Comp. Endocr. 114, 2-10.

Introduction

The enteropneusts, with the pterobranchs and planktosphaeroid larvae, comprise the small deuterostome phylum Hemichordata, generally regarded as an early offshoot of the chordate line of evolution (Ruppert and Barnes 1994). Several authors have described a distinctive type of epidermal gland cell in enteropneusts termed the `mulberry' cell from its berry-like cluster of apical granules (Brambell et al., 1939; Hyman, 1959; Knight-Jones, 1952; Welsch, 1984). Such cells have been reported under other names, for instance "granular gland cell" (Bullock and Horridge, 1965) and "elongated gland cell" (Dawydoff, 1948). Schneider (1902) called them "protein cells" from the reaction of the granules with haematoxylin and aniline dyes. All authors agree in distinguishing mulberry cells from the more common mucous cells, but there may be other densely staining granular cells present which could cause confusion (see Welsch, 1984). Pardos and Benito, (1989) for example describe mulberry-like cells ("coarse grain cells") in the endoderm of the pharynx but as these are ciliated they cannot be equated with mulberry cells, which lack cilia.

As described by Brambell et al. (1939) in *Saccoglossus cambrensis* the cells are oval or pear shaped, measuring 20 μm in length and approximately 7 μm at the thickest part. The cells have a narrow basal process that runs down and mingles with the mass of intraepidermal nerve fibres lying over the basal lamina. This basal process resembles a neurite and the mulberry cell may therefore be a type of neuron (Bullock, 1945) but physiological evidence of neural function is lacking. The only two electrophysiological studies so far carried out on enteropneusts (Pickens, 1970; Cameron and Mackie, 1996) were both restricted to extracellular recordings and provide no information on the activity of individual excitable units.

We will show in this paper that the mulberry cell granules are immunoreactive with antisera against gonadotropin-releasing hormone (GnRH, previously termed luteinizing hormone-releasing hormone, LHRH). The best-known function of this important reproductive hormone in vertebrates is to elicit the release of the pituitary gonadotropins. GnRH has been identified by primary structure in each class of vertebrates, where the effect of GnRH release on reproduction has been investigated (see Sherwood et al., 1993). Peptides of the GnRH family have N-termini modified to form a pyroglutamyl ring and conserved amino acid residues in positions 1, 2, 4, 9 and 10. This conservation of form has recently been extended to include a tunicate, the ascidian *Chelyosoma productum* (Powell et al., 1996). There is one report of a form of GnRH that coelutes with mammalian GnRH in a mollusc (Goldberg et al., 1993), challenging the view that GnRH is restricted to the chordate lineage.

Immunocytochemical evidence alone cannot provide conclusive evidence of the presence of a particular peptide. In the tunicate work, the initial identification of a GnRH-like peptide (Georges and Dubois, 1980) was later extended by column chromatography

(Dufour et al., 1988) and high performance liquid chromatography (HPLC) (Kelsall et al., 1990) showing that GnRH was indeed present. By the same token, satisfactory demonstration of the presence of GnRH in *Saccoglossus* requires chromatographic evidence as well as data from immunocytochemistry. We therefore embarked on this project using both techniques.

Materials and Methods

Two species of the acorn worm *Saccoglossus* occur in coastal waters of British Columbia and Washington, *S. bromophenolosus* from Willapa Bay and Padilla Bay, Washington (King et al. 1994) and a species provisionally designated *Saccoglossus* species A, pending determination by a specialist, from Barkley Sound, British Columbia. They differ in size, coloration and habitat. *S. bromophenolosus* lives intertidally in mud flats and can be collected during periods of low tide while *S.* species A typically occurs subtidally in coarse, calcareous sediment of biogenic origin and was collected by SCUBA. Immunolabelling for GnRH was carried out on both species but *S.* species A provided the preparations used for the photographs in this paper and was also selected for sectioning for optical and electron microscopy. GnRH extraction requires large numbers of animals, so the more easily collected *S. bromophenolosus* was chosen for this work. Specimens of both species were collected during spring and summer 1994, and were transported to the University of Victoria where they were maintained in their natural substrates in slowly running sea water at 12 ^{o}C. More specimens of *S. bromophenolosus* were collected in the same season in 1995, but were frozen whole in liquid nitrogen at the collection site. Specimens of *Ptychodera bahamensis* (Enteropneusta: Ptychoderidae) and *Cephalodiscus gracilis* and *Rhabdopleura normani* (Pterobranchia) were obtained intertidally at Castle Beach and under the Causeway Bridge, Bermuda, and were studied at the Bermuda Biological Station for Research in March, 1996.

Immunocytochemistry. After anesthesia in sea water containing 151 mM Mg2+ specimens were dissected and pieces of tissue were removed and pinned out in Sylgard-lined petri dishes where they were fixed for 24 hours in Zamboni's fixative (Zamboni and DeMartino, 1967) at pH 7.3. After washing in phosphate buffered saline (PBS) the tissues were treated with the primary (rabbit) antibody diluted 1: 500 in PBS + 3% Triton X100 + 1% goat serum . Tissues were washed repeatedly in PBS containing 0.3% Triton X100 for 18 hours and treated with FITC-labelled goat anti-rabbit gamma globulin (FITC-GARGG) diluted 1:500 in PBS + 3% Triton X100 for 60 min. Tissues were washed again in PBS and mounted as whole mounts in 50% glycerol containing 1.5% N-propyl pyrogallate. Two primary antisera were used: rabbit anti-mammalian GnRH, sample U705-23, from Dr. Gerald Kozlowski (University of Texas) and rabbit anti-salmon GnRH, sample GF4, from N.M.

Sherwood (University of Victoria). In addition, an antiserum against Tunicate-1 GnRH was used in the experiments conducted in Bermuda. Both the primary antibodies and FITC-GARGG were used at a dilution of 1:500. Two types of controls were run. In the first, the primary antibody was omitted during the initial incubation. In the second, the preparations were run with primary antisera preabsorbed for 24 hours with mammalian GnRH.

For visualizing nuclei in the mulberry cells, the fluorescent Hoechst dye # 33342 (Sigma) was used on Zamboni-fixed whole mounts.

Sections. Specimens were fixed in Bouin's fluid for paraffin sectioning. Four specimens were serially sectioned from the proboscis through the collar to the anterior part of the trunk. The sections were stained with eriochrome cyanin.

Pieces of tissue were fixed in 2.5% glutaraldehyde in 0.2M Millonig's phosphate buffer at pH 7.4 for 1.5 hrs. They were post-fixed in 1% osmium tetroxide in 0.2M Millonig's buffer for 1 hr and dehydrated through a graded series of ethanol solutions followed by propylene oxide and embedded in Epon 812. Thick (ca. 1.0μm) sections were cut with glass knives and stained with Richardson's stain for light microscopy. Thin sections, stained in uranyl acetate and lead citrate, were examined with a Hitachi H-7000 transmission electron microscope.

Extraction of peptides. Approximately 1200 *Saccoglossus bromophenolosus* were collected in 1994 from intertidal sediments of Willapa Bay West, Washington, and transported in sediment and sea water to aquaria at the University of Victoria. The probosces and collars (73.2 g) were dissected, immediately frozen on dry ice and stored at -80 °C. Frozen tissue was powdered with liquid nitrogen in a Waring Blendor. The powdered material was treated as described by Sherwood et al. (1986). Briefly, the material was added to 1N HCl/acetone (3:100 v/v), stirred for 3 h and filtered through a #1 Whatman filter. The solids were resuspended in 0.01N HCl/acetone (1:5 v/v) and stirred for 3min. Acetone, lipids and other hydrophobic substances were removed by five successive additions of petroleum ether (20% v/v). The final aqueous phase (800 ml) was evaporated in a vacuum centrifuge to approximately 200 ml. An additional 3000 specimens of the same species were collected at the same location in 1995. The acorn worms (575 g) were frozen whole in liquid nitrogen at Willapa Bay, then taken to the University of Victoria.

Sep-Pak high performance liquid chromatography (HPLC). Ten Sep-Pak C18 cartridges (Waters) were connected in series and washed with 6 ml of methanol followed by 6 ml of Milli-Q water. The aqueous extract from the tissue sample was pumped through the cartridge column using a peristaltic pump at a flow rate of 1.5 ml/min. The material remaining on the column was eluted after the cartridge column was connected to a Beckman Model

125 HPLC apparatus. Initial conditions of solvent flow in the column were 95% solution A (0.05% trifluoroacetic acid, TFA, in water) and 5% solution B (0.05% TFA in 80% acetonitrile and 20% water) at a flow rate of 1 ml/min. A gradient that increased at a rate of 1% solution B per minute was applied to the cartridge column for 60 min. Fractions of 1 ml were collected for 60 min and assayed for GnRH-like immunoreactivity. Specimens collected in both years were extracted by the same method.

Purification of GnRH. Procedural steps for the purification of a GnRH-like peptide included three successive HPLC stages after Sep-Pak HPLC. A new C18 Supelco column connected to a Beckman 125/166 HPLC and detector was used. Solvents and ion-pairing agents for the HPLC steps are listed in Table 1. The last step of the purification was done with a phenyl column (Vydac) to determine if more than one GnRH-like peptide could be separated. Aliquots of 100 µl were used to determine the amount of immunoreactive GnRH (irGnRH) in each fraction collected. Fractions that contained irGnRH were selected for further purification in successive steps.

In most GnRH purification schemes, some of the immunoreactive GnRH elutes early (fractions 1-10) without interacting with the column. This occurred also in the *Saccoglossus* GnRH purification. With the extract prepared in 1995 several methods were designed to improve the yield of GnRH. To disrupt any weak protein binding to carrier molecules and allow stronger interaction (retention) with the C-18 column, pooled fractions of early-eluting material were treated with a 6M guanidine HCL solution prior to column loading. This treatment did not alter the elution position. A stronger ion-pairing agent, 0.05% hepta-fluoro-butyric acid (HFBA), was then used in the HPLC program and similar results were obtained. Finally, a polyhydroxy-ethyl aspartamide column (200 x 4.6 mm, PolyLC Inc. Columbia, MD) was tested for use in purification of the early eluting fractions using hydrophilic interaction chromatography (HILIC). HPLC fractions 1-10 were vacuum dried to 0.5 mls, then diluted with a 20% methanol/80% acetonitrile solution to a final volume of 3 mls. Five consecutive 600 µl volumes were injected into the column (at 2 min intervals) and eluted using the following elution profile: 100% B for 10 min (A=0.05% TFA, B=0.05 TFA/90% acetonitrile), 85.5% B for 10 min, then 85.5% to 54% during a 40 min period.

Radioimmunoassay (RIA). Two identical RIA methods were used except that the labeled trace was mammalian GnRH in one method and lamprey GnRH-I in the other method. Iodination methods were identical for both GnRH peptides. Antisera GF-4, BLA-5 and R-42 were used in the mammalian GnRH method and antisera 36-52 and 7CR-10 were used in the lamprey GnRH-I method. The standard was mammalian GnRH for the first three antisera, lamprey GnRH-I for the 36-52 antiserum and chicken GnRH-II for the 7CR-10 antiserum. An aliquot of each HPLC fraction was assayed for irGnRH by standard RIA (Sherwood et al. 1983,1986).

The cross-reactivity of GF-4, BLA-5 and R-42 have been previously reported (Kelsall et al., 1990; Sherwood et al., 1991). Antiserum GF-4 (raised against salmon GnRH) was used in a dilution of 1:25,000 resulting in 22-32% binding of ^{125}I-mammalian GnRH. Antiserum BLA-5 (raised against lamprey GnRH-I) was used in a dilution of 1:10,000 resulting in 9-17% binding of ^{125}I-mammalian GnRH. Antiserum R-42 (raised against mammalian GnRH) was used in a dilution of 1:50,000 resulted in 10% binding of ^{125}I-labeled mammalian GnRH. All of the known GnRH forms are recognized by at least one of these antisera. Limits of detection (B/Bo=80%) for each assay averaged 10.4 pg for GF-4 and 47.6 pg for BLA-5.

Antiserum 36-52, raised against lamprey GnRH-III, was a gift from Dr. Stacia Sower (University of New Hampshire). Antiserum 36-52 was used in a dilution of 1:25,000 and resulted in 42% binding of ^{125}I-labeled lamprey GnRH-I. The limit of detection was 33 pg. The cross-reactivity of 36-52 was 100% for lamprey GnRH-III. Antiserum 7CR-10 (raised against dogfish (df) GnRH) was used in a dilution of 1:37,500 resulting in 12% binding of 125I-labeled lamprey GnRH-I. The limit of detection was 22 pg. The cross-reactivity of 7CR-10 is 100% for cGnRH-II, 25% for dfGnRH, 6% for lamprey GnRH-I and under 0.03% for other known forms of GnRH.

Results

Mulberry cells in Saccoglossus: structure and immunolabelling. As noted above (p 2), these cells have been described by several previous workers and are readily identified by their prominent granular contents, long basal processes and lack of cilia. The apical end of the cell forms part of the epithelial surface while the basal process runs down into the mass of nerve fibres lying over the basal lamina. The nucleus lies just below the mass of granules in the part of the cell that tapers down to form the basal process. In paraffin sections and in thick Epon sections after staining with basic dyes the granules are strongly basiphilic. They are electron dense under the electron microscope after osmium staining. In both species examined, the granules label strongly with anti-GnRH antisera (Fig 1A, B). No labelling was observed in the basal processes. No GnRH-like immunofluorescence was detected in control preparations.

Seen by electron microscopy fully differentiated mulberry cells could be followed from their apical poles down to their narrow basal processes in the nerve fibre layer (Fig 1D) close to the basal lamina. At the apical pole, granules appear to be undergoing release to the exterior (Fig 1C). There were typically about 30-45 granules per cell, the granules showing a mean diameter of 970 ± 280 nm (n=62). These measurements are in agreement with measurements made on the granules in immunolabelled whole mounts. The narrow basal processes extending into the fibrous layer mingle with the processes of neurons and neurosensory cells and cannot readily be distinguished from the latter either in terms of size or cytoplasmic contents. The processes lying in the fibre layer contain numerous microtubules and mitochondria. Small clear vesicles and various sizes of dense-cored vesicles are seen in many processes (Fig 1D). It has not been possible to trace the basal processes of mulberry cells far enough in the fibrous layer for their lengths to be estimated and it is not known if they make synaptic interconnections with other processes.

Mulberry cell distribution in Saccoglossus. Observed in immunolabelled whole mounts mulberry cells were found widely distributed through the body wall ectoderm in the proboscis, collar and anterior part of the trunk (Fig 2). In the proboscis they were particularly abundant ventrally to the neural keel in the proboscis neck and adjacent to the proboscis pore. In the collar, they were abundant in the anterior collarette epithelium and in the anterior part of the collar nerve cord but were rarely observed in the internalized part of the collar cord. Where the cord surfaces again at the back of the collar, mulberry cells were again found. In the trunk, mulberry cells were seen as lines of cells following the gill slit primary and tongue bars. Mulberry cells were not observed elsewhere in the trunk. In all regions where they were observed, the mulberry cells were associated with nervous tissue, and sent their basal processes into the nerve fibre layer.

Mulberry cells in other hemichordates. Mulberry cells were located in *Ptychodera behamensis* using antisera against tunicate-1 GnRH and mammalian GnRH. The cells showed a distribution similar to that described above for *Saccoglossus.* No immunoreactive cells were found in the pterobranchs *Cephalodiscus* and *Rhabdopleura* using these antisera.

Characterization of Saccoglossus GnRH. Of the five antisera used in RIA, antiserum 36-52 combined with 125I-labeled lamprey GnRH-I detected irGnRH from a C-18 column in HPLC eluates from *Saccoglossus bromophenolosus* extract. In contrast, as noted above, GF-4 detected irGnRH in whole mounts of both species studied. A total of 2.7 ng irGnRH was detected in fractions 33-40 of Sep-Pak HPLC (Fig 3A). These fractions were combined, reduced in volume and applied to the isocratic-TEAF HPLC method where eluates in fractions 19-25 contained a total of 3.3 ng irGnRH (Fig 3B). In further purification using the gradient-TFA method a total of 0.3 ng of irGnRH was detected in fractions 22-24 (Fig 3C). After application of these fractions to the phenyl column with TFA in the mobile phase, 0.3 ng of irGnRH was detected in fractions 22 and 23 (Fig 3D).

Elution of the irGNRH material from the hydrophilic column resulted in several small peaks. GnRH-like material was detected using anti-lamprey (36-52) in HILIC fractions 4 (0.4 ng), 25, 31-32 (0.64 ng) and 44 (0.31 ng). Antiserum R-42 also detected immunoreactive GnRH in fractions 25, 31-32 and 44. Tunicate GnRH-I antiserum detected GnRH immunoreactivity in fractions 8, 24,32 and 48, whereas antiserum 7CR-10 detected irGnRH only in fraction 44. The immunoreactive material was purified further, but insufficient material was available for determination of sequence or mass.

Discussion

A close phylogenetic relationship between hemichordates and chordates, first suggested by Bateson (1885), is now widely accepted. Some evidence from molecular biology (Holland et al., 1991) supports the idea of the hemichordates as an early offshoot from the chordate line of evolution. The finding of GnRH in enteropneusts provides further support for this view as GnRH is prototypically a chordate line hormone. Other studies link the group with the echinoderms (Wada and Satoh, 1994; Turbeville et al., 1994; Halanych, 1995). It now becomes highly desirable to obtain the amino acid sequence for saccoglossan GnRH for the evidence it may provide regarding early evolution of this important molecule in the chordate lineage.

The characterization of this irGnRH-like peptide by HPLC-RIA is not unlike the HPLC elution pattern seen in the purification of other vertebrate and invertebrate GnRH peptides by this method (Sherwood et al., 1986; Powell et al., 1995). Indeed, the presence of at least one GnRH peptide is supported by the repeated application, elution and detection of irGnRH from a new C18 HPLC column in repeated HPLC procedures. The detection of GnRH-like immunoreactivity with several antisera following hydrophilic interactive chromatography strengthens the hypothesis that GnRH-like material exists in *Saccoglossus* and suggests that more than one form of GnRH may exist.

Further, this study parallels the work by Kelsall and coworkers (1990) who detected irGnRH by immunohistochemistry and in HPLC eluates of the ascidian *Chelyosoma productum*. This material was later identified by primary structure (Powell et al., 1996). The detection of only one form of irGnRH in HPLC eluates of *Saccoglossus* suggests either that additional GnRH peptides were not detected by the antisera or that there is only one form of GnRH in hemichordates. The presence of two distinct GnRH peptides in *Chelyosoma* may mean that a gene duplication occurred in ancestral tunicates after they separated from hemichordates. Until *Saccoglossus* GnRH is sequenced, such evolutionary questions cannot be answered.

In vertebrates and ascidians, GnRH is typically a secretion product of neurons. Thus it is not surprising to find that in *Saccoglossus* and *Ptychodera*, irGnRH is found only in the mulberry cells, which are probably neurons. The anatomy of the mulberry cell is suggestive of a neurosecretory cell that releases its contents to the exterior. If so, the peptide would presumably mix with the mucus covering the skin, or diffuse into the surrounding sea water. In vertebrates and ascidians by contrast the cytological evidence is entirely consistent with a purely endocrine role and there is no reason to suppose that GnRH is liberated to the exterior. The basal neurites of the mulberry cells show little if any irGnRH, which suggests that the peptide is not used as a transmitter or modulator at synapses in the fibre layer and is not released internally as a hormone. On the basis of present evidence we therefore propose an exocrine (possibly pheromonal) role for saccoglossan GnRH.

Table VI-1. Steps in the HPLC purification of GnRH from *Saccoglossus bromophenolosus*. Solvent and column types are listed for each successive step. Immunoreactive areas identified by radioimmunoassay were reduced in volume, combined and applied to the next step of purification.

HPLC Step	Column Type	Solvent A	Solvent B
1	Sep-Pak	0.05% TFA	0.05% TFA in 80% ACN/20% H20
2	C18	1.2mM TEAF	ACN
3	C18	0.05% TFA	0.05% TFA in 80% ACN/20% H20
4	Phenyl	0.05% TFA	0.05% TFA in 80% ACN/20% H20

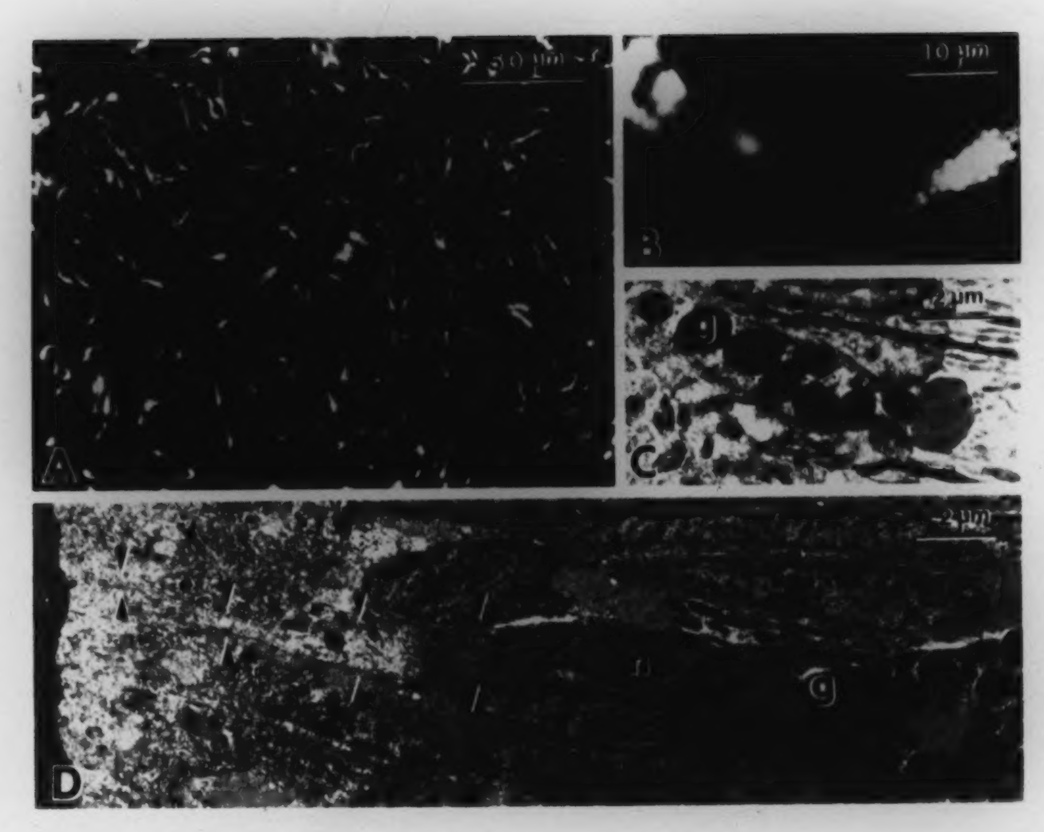
Abbreviations:

ACN: acetonitrile

TEAF: triethylammonium formate

TFA: trifluoroacetic acid

Figure. VI-1. Mulberry cells in *Saccoglossus* shown by immunofluorescence (A,B) and transmission electron microscopy (C,D). A) GnRH-like immunoreactivity in mulberry cells scattered through the posterior collar epithelium, dorsal to the collar nerve cord. B) higher magnification, cells extending from the dorsal collar nerve cord. C) apical portion of a mulberry cell in the mid-collar region, with granules. D) transmission electron micrograph showing granules (g), nucleus (n) and tapering base of mulberry cell running down into nerve fibre layer (arrowheads).


50 µm
10 µm
2 µm
2 µm
A
B
C
D
g
n
g

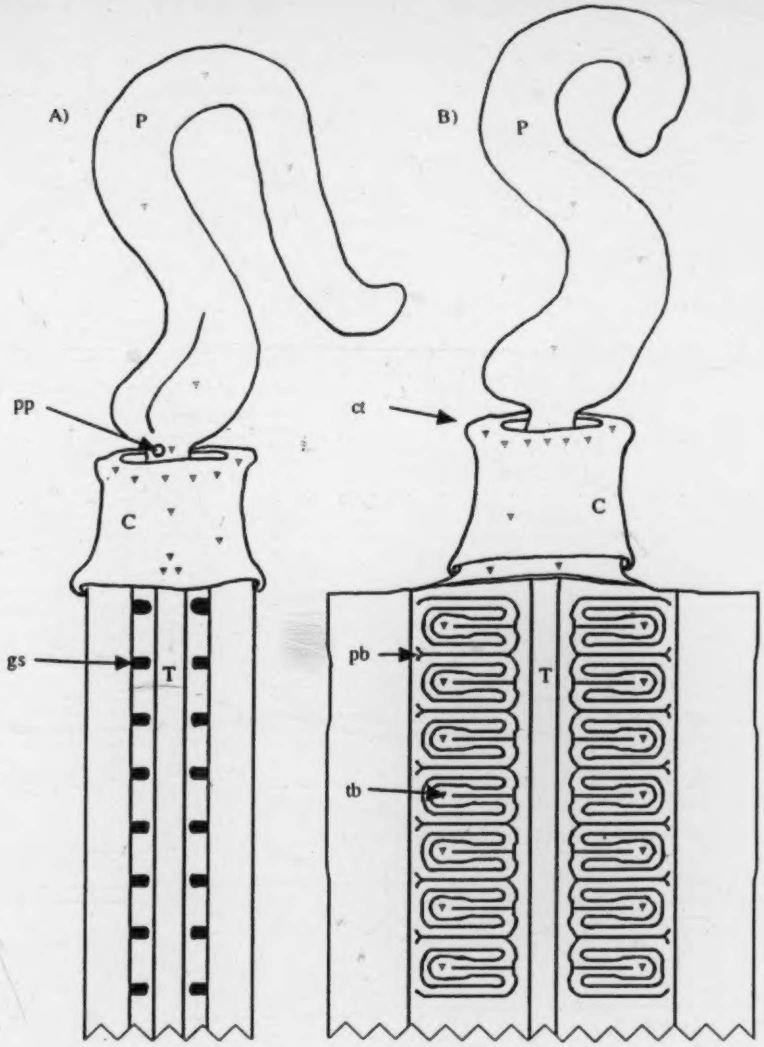


Figure. VI-2. The distribution of GnRH immunoreactive mulberry cells in *Saccoglossus*. A) dorsal view showing mulberry cells in the general proboscis epithelia with incresing density in the region of the gill pore, collarette, and overlaying the dorsal collar cord, B) ventral view of the mulberry cell around the collarette, and with the trunk segment cut mid-ventrally to show the distribution in the gill bar epithelia. Not to scale. collar (C), collarette (ct), gill slit (gs), primary gill bar (pb), proboscis (P), proboscis pore (pp), tongue gill bar (tb), trunk (T), mulberry cell (*).

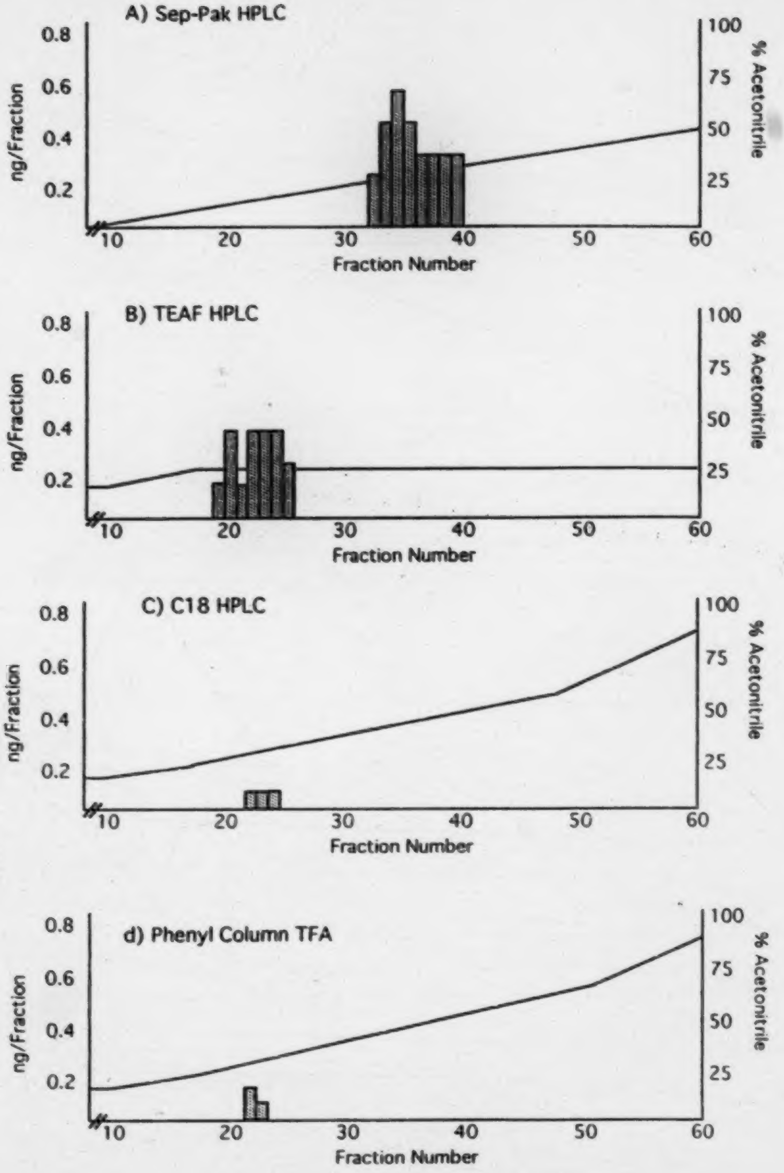


Figure. VI-3. HPLC analysis of immunoreactive GnRH from *S. bromophenolosus* extract. A) irGnRH in eluates from a Sep Pak column with TFA in the mobile phase, B) irGnRH in eluates from a C18 column with TEAF in the mobile phase, C) irGnRH in eluates from a C18 column with TFA in the mobile phase, D) irGnRH in eluates from a phenyl column with TFA in the mobile phase. Solid lines indicate % acetonitrile in the mobile phase.

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CHAPTER VII

Particle retention and flow in the pharynx of the enteropneust worm *Harrimania planktophilus*: The filter feeding pharynx evolved prior to the divergence of the hemichordates and the chordates

Abstract An investigation of the feeding behavior of the acorn worm *Harrimania planktophilus* suggests a wholly novel form of enteropneust feeding that has significant phylogenetic implications. *H. planktophilus* is a holoinfaunal worm that appears to feed on both deposited sediments and suspended particles in interstitial pore water. To visualize the particle retention behavior involved in filter feeding, adult animals were held in chilled sea water under low light and fed food coloring and fluorescent particles while being video recorded. Most particles ingested were drawn into the mouth by an incurrent created by cilia on the pharyngeal bars and without the aid of mucus. Particles that passed freely through the gill pores averaged 3.04 µm whereas particles retained in the gut and defecated in the feces averaged 13.9 µm. Food coloring entered the mouth and was pumped through the pharynx at a rate of 0.5 - 2.0 mm/s. There was no evidence of an endostyle or mucus-net capture mechanism in *H. planktophilus*, but instead particles were filtered and manipulated by a dense covering of cilia on the pharyngeal bars. This study suggests that the suspension feeding pharynx is not an innovation of the chordates, but evolved prior to the evolutionary divergence of the hemichordates and chordates.

Introduction

The worm-like enteropneusts and bryozoan-like pterobranchs comprise the small deuterostome phylum Hemichordata, generally regarded as an early offshoot from the chordate line of evolution (Ruppert and Barnes 1994). Such a group might be expected to hold clues concerning early chordate pharyngeal evolution, but pharyngotremy and feeding behavior of hemichordates have been little studied since the work of Barrington (1940; Barrington and Thorpe 1963). What little work has been done on enteropneust feeding behavior has primarily focussed on deposit feeding and so the Enteropneusta have long been believed to be classical muco - ciliary feeders, collecting their food particles on the proboscis with mucous secretions and transporting them back to the mouth using multiciliated cells (Burdon - Jones 1962, Knight - Jones 1953, Barrington 1940, Thomas 1972). Although some evidence of suspension feeding has been observed in *Balanoglossus gigas* (Burdon-Jones 1962), *Saccoglossus horsti* (Knight - Jones 1953), and *Glossobalanus minutus* (Barrington 1940), it has been regarded as of secondary (Burdon - Jones 1962, Knight - Jones 1953) or of minor importance (Thomas 1972).

Most of what we know about filter feeding in the Enteropneusta is from the research of Barrington (1940), Knight - Jones (1953) and Burdon - Jones (1962). Barrington (1940) studied the paths that carmine particles took in and around the collar lip of *Glossobalanus minutus*. The majority of particles were transported with the aid of mucus, but some were pulled into the mouth with a ciliary current, suggesting that enteropneusts were able to acquire food by suspension feeding. No particle size measurements were made in that feeding experiment and so clearance rates of suspended particles by *G. minutus* can not be estimated. Respiratory currents have been observed to enter the mouth of burrowing *Saccoglossus horsti* (Knight-Jones 1953), and apparently assist sediment-laden mucus to enter the mouth (Knight-Jones 1953).

Balanoglossus gigas, the largest of the enteropneusts, grows to lengths exceeding two meters (Horst 1939) and creates deep burrow systems with many connections to the surface (Burdon - Jones 1962). Burdon - Jones (1962) released graphite suspensions at the margin of the collarette of *B. gigas*. Graphite particles were swiftly drawn into the dorsal pharynx, but there was no indication of the role of mucus. Particles were trapped on the branchial arches and then transported postero-ventrally and accumulated in shallow grooves at the base of the parabranchial ridges (which separate the dorsal branchial pharynx from the ventral digestive pharynx). The finer suspended particles were drawn into the gill slits, sieved off by the synapticles and transported slowly ventralwards. Ultimately the graphite (at this point trapped in mucus) from the arches and slits were rolled into a loose cord and transported slowly posterior and ventrally into the ventral digestive digestive gut. This sieving mechanism was very efficient and prevented particles 1 to 2 µm from passing out through the branchial pores (Burdon - Jones 1962).

Among deuterostomes, gill slits occur in both hemichordates and chordates. In enteropneusts they are generally believed to dispose of excess water that is transported in the pharynx with ingested sediment (Barrington 1940, Barrington and Thorpe 1963, Knight - Jones 1953, Burdon - Jones 1962) and assumed to be gas-exchange surfaces (Ruppert and Barnes 1994). The chordate pharynx, on the other hand, orchestrates the collection of food (ascidians and amphioxus), and provide a means of locomotion for salps and doliolids (Bone 1998). The pharynx of lamprey ammocete larvae functions much like that of amphioxus, and in the vertebrates the pharynx functions as a surface for gas exchange (although suspension feeding has been reinvented in some taxa). In higher vertebrates the gill bars have been modified to form the jaws and the hyoid arches (Radinsky 1987).

Given the unquestionable shared form of the hemichordate pharynx with that of lower chordates, and the paucity of information on enteropneust suspension feeding, pharyngeal structure, and behavior, I decided to investigate *Harrimania planktophilus* (Cameron) from Barkley Sound, British Columbia. My goals were (i) to confirm preliminary observations suggesting suspension feeding by this enteropneust, (ii) to quantify the particle size range that the pharynx can filter from sea water, and (iii) to carry out a microscopical examination of the pharynx to better understand the movement of water and particles through the pharynx.

Materials and Methods

Harrimania planktophilus was collected from the intertidal sand flats at Cape Beale (48° 47' 30" N, 125° 12' 56" W) Barkley Sound, Vancouver Island, Canada. Twenty one worms were collected over the summer of 1999 and transported back to the Bamfield Marine Station where they were kept in specimen bowls containing their natural sediment under a flow of fresh sea water. One or two worms at a time were removed from their bowls for either video micrography or for light and SEM microscopy. For the purpose of videotaping the feeding behavior of *Harrimania planktophilus*, animals were first placed in finger bowls without sediment, and then allowed to cool on ice until the water temperature was between 5 and 7 °C. Cooling the water was the most effective way to relax the muscular and secretory responses of the animals in response to the stress of being removed from the sediment. An ice bath was also the most effective means of keeping the water cool while filming. *H. planktophilus* is strongly photonegative, and therefore animals were filmed with the lowest light levels possible while still maintaining a visible image. An orange, red and blue filter placed between the fibre optic light source and the animal did not appear to reduce their photosensitivity. To image the current through the mouth and pharynx of *H. planktophilus*, a number of particles suspended in sea water were tried, including: carbon dust, starch, sephadex beads, carmine red, and fluorescent particles (Dayglo Color Corp.). Dyes in sea water solution were also tried, including: diluted milk, methylene blue, fluorescein, and blue food coloring. *H. planktophilus* responded most positively to fire orange fluorescent particles, series A (Dayglo Color Corp.) and blue food coloring in sea water. The fire orange fluorescent particles were positively charged and had a size range of 1.6 - 20 µm, with 20% (the mode) at 6um. Quantification of particle size cleared and captured by *H. planktophilus* was determined by direct measurement of particles that passed freely through the gill slits and particles that were bound into fecal material. Feeding trials were filmed and measurements taken with a JVC 3-CCD colour video camera on a WILD dissecting microscope. Still pictures in figures 1 and 2 were captured with Optimus (Optimus Corporation) software.

Animals prepared for light microscopy were allowed to defecate their gut contents and cleaned externally of sediment before relaxing in 7% $MgCl_2$ followed by fixation in Bouin's solution and dehydrating through a graded series of ethanol. Once in 100% ethanol animals were dissected into small pieces, transferred to xylene, followed by infiltration of paraffin wax. Sections were cut on a American Optical Corporation "820" Spencer microtome and stained with either Delafield's hematoxylin or eriochrome - cyanin, and viewed and photographed with an Olympus OM-4T 35 mm camera on a Olympus BH2 compound microscope. For scanning electron microscopy pieces of tissue were dissected from a relaxed animal so that the dorsal pharynx was exposed. The specimen was then fixed in 2.5% glutaraldehyde in 0.2M Millonig's phosphate buffer at pH 7.4 for 1.5 hrs and post-fixed in 1% osmium tetroxide in 0.2M Millonig's buffer for 1 hr. The specimen was then dehydrated through a graded series of ethanol solutions, followed by critical point drying and spatter coating with colloid gold, and examined with a Hitachi S-3500N scanning electron microscope.

Results

Histology and microstructure

Sections were cut through the pharynx and particular attention was paid to the dorsal branchial pharynx (Fig. 1E), where the feeding current was presumed to be maintained in *Balanoglossus gigas* (Burdon - Jones 1962). Long cilia were observed on the lateral side of the primary and secondary gill bars (Figs. 1C & F), and at the pharyngeal edge of the atrial sac (Fig. 1F). None of the cilia showed evidence of modified tips. Scanning electron micrographs revealed no synapticles bridging the secondary gill bars to the primary gill bars, and consequently the tongue bars project far into the pharynx lumen (Fig. 1 A). The pharyngeal region of *H. planktophilus* was 5 times the length of the extended proboscis, and had 36.2 ± 9.7 (N=6) pairs of large gill slits that connected to muscular gill pores which were located in paired dorso-lateral grooves on the outside of the body. In some animals the gill skeletons were pigmented dark black, rather than the usual collagenous opaque white color, allowing them to be easily visualized through the body wall. The post-pharyngeal region had a large bilobed thickening of the dorsal gut epithelium (Fig. 1D), the post - pharyngeal organ. In live animals the post -pharyngeal organ was pigmented brick red, as observed through the body wall. It presumably was involved in squeezing the excess water from the food before being transported to the digestive gut. Shortly after the post-pharyngeal organ the gut became darkly pigmented and sinuous, presumably to increase the surface area for digestion and absorption. Shortly before the anus the gut began to straighten. The anus had a sphincter muscle.

Most of the suspended material appeared to pass into the dorsal chamber of the branchial pharynx (Fig. 1A & E), where it was filtered by heavily ciliated gill bars. Mucus was observed in the pharynx (Fig. 1B & C) and on the proboscis epithelium (Fig. 1E), and possibly aided the collection and transport of food particles. Flourescein particles were transported ventrally down the gill bars and collected into neat masses at the ventral tip of the tongue bars before being passed on by ciliary action towards the post-branchial chamber. Enteropneusts that were fed particles in suspension for more than a few minutes would slow their feeding rates and eventually stop feeding altogether because the pharynx would become plugged with particles. The 'bottle neck' in the particle filtration system was at the post-pharyngeal organ (Fig. 1D) and in the digestive pharynx. The branchial pharynx could therefore capture a wide range of particles at a rate that exceeded the processing efficiency of the gut.

Observations of flow

Fluid velocity through the mouth was estimated from video recordings of blue food coloring (Fig. 2) that was introduced around the mouth with a hand held pipette (Fig. 2B). The average rate of flow into the mouth and through the pharynx was about 0.5 - 2.0 mm/s. *H. planktophilus* regulated its feeding flow in three ways: 1) by contracting the gill pore sphincter muscles, 2) by sealing off the mouth with the posterior proboscis, or by sealing the purse-string-like collar lip around the neck, and 3) by ciliary arrest in the branchial pharynx. The feeding flow through the mouth of *H. planktophilus* was stopped by ciliary arrest when the animal began to burrow, or when the animal had a pharynx full of particles.

At these times the mouth and gill pores were wide open, yet a feeding current was not observed. When the worm bent the body to turn right, the gill pores on the inside (right) side closed and water exited only from the left pores, and vice-versa. A muscular sphincter, derived from the longitudinal trunk musculature, curves around each pore (Horst 1939). Reynolds numbers were approximately 10 through the mouth (~0.5 mm diameter) and therefore water flow was nearly laminar. *H. planktophilus* was continually active and consequently filtered water would rarely be reingested.

Particle retention

The size of particles that passed freely through the gill slits (Fig. 3) and pores of 6 worms measured 3.04 µm ± SD 1.52 µm (N = 61), the maximum particle size was 5.8 µm, and the minimum was 0.2 µm. Particles collected from the feces had a mean size of 13.9 ± SD 8.6 µm (N = 48) with a minimum size of 0.2 µm and a maximum of 28.4 µm. Particles in the feces were compacted in the gut, and packaged in a mucous matrix resulting in a tubular fecal casting. Particles had to be teased from the fecal casting for measuring and therefore the large size range may have been overestimated due to multiple particles adhering together or underestimated because the larger sizes may have been broken up in the gut or while I was manipulating the feces. Therefore particle sizes measured in the feces may not be a precise indicator of those that entered the mouth. Despite the problems incurred with particle size estimations, I am confident that *H. planktophilus* is able to ingest particles from the entire size range (0.2 µm – 30 µm) offered by the positively charged fluorescent thermoplastic particles, series A (Dayglo Color Corp.) and that the largest size that may pass through the gill slits, atria and gill pore is 5.8 µm, suggesting that *H. planktophilus* is a suspension feeder that exploits a wide size range of particles available in sedimentary pore water.

Food in the form of sediment, detritus and plankton was also trapped in mucus on the proboscis and transported posteriorly over its surface toward the mouth. The pre-oral ciliary organ (Fig. 3A), located on the posterior face of the proboscis, worked as an effective sorting organ to shunt unwanted particles dorsally where they were transported rearward by cilia over the dorsal collar lip and trunk, or shunted to the ventral midline where they were carried to the mouth on a string of mucus. Passage time through gut was about 5 hours in a 3 cm long worm.

Discussion

Harrimania planktophilus appears to have a feeding behavior similar to that described in protochordates, suggesting that a filter-feeding pharynx evolved before the divergence of the hemichordates and chordates. Long cilia, located on the lateral sides of the gill bars and in the atrial cavity, probably mediate the pumping behavior, and capture most of the particles from suspension, but a fuller experimental study would be necessary to demonstrate this conclusively.

Burdon - Jones (1962) described a ciliary current within the collar and the anterior region of the pharynx of *Balanoglosus gigas*. Particles entering the pharynx of dissected animals are initially trapped by the dorsal, branchial pharynx and then transported ventrally to the digestive pharynx en route to the gut. The observations of particle movement in the present study were made through the body wall and closely resemble those reported by Burdon - Jones (1962). In neither case was there any indication of a mucous net associated with the pharynx like that of the invertebrate chordates. Instead, heavily ciliated gill bars appear to secrete mucus. Except for the long cilia mentioned above, cilia in the pharynx were unspecialized and none exceeded about 8 µm in length. Such cilia are evidently sufficient for pumping and filtration of food-laden water.

The observations above agree with those of Burdon - Jones (1962) who found particles of 1 - 2 µm retained by the gill slits of *B. gigas*. *Harrimania planktophilus* retained particles as small as 0.2 µm and as large as 28.4 µm in the pharynx, although the filter is not completely efficient as particles as large as 5.8 µm passed freely through the gill pores. In any case, the size range of particles captured in this enteropneusts pharynx exceeded those of the ascidians *Ascidella asperense*, *Molgula manhattensis*, *Clavelina lepadiformis* and *Ciona intestinalis* (Randlov and Riisgard 1979).

Recent experiments reveal that key developmental genes are expressed in the epithelia of differentiating gills of both ascidians and the enteropneust *Ptychodera flava* (Ogasawara et al. 1999), suggesting that the gill slits of enteropneusts are homologous to those of chordates. A test of this idea might be to look for the expression of members of this gene family in the pharyngeal pores of macrodasyid gastrotrichs (Ruppert and Barnes 1994), to see if the gene is responsible for pores or openings in the pharynx in general, rather than having an expression that is specific to deuterostome gill pores. The gill slits of enteropneusts and chordates appear to be homologous based on their shared location, developmental origin and, at least in *H. planktophilus*, their shared function. How then do the gill arches fit into this picture?

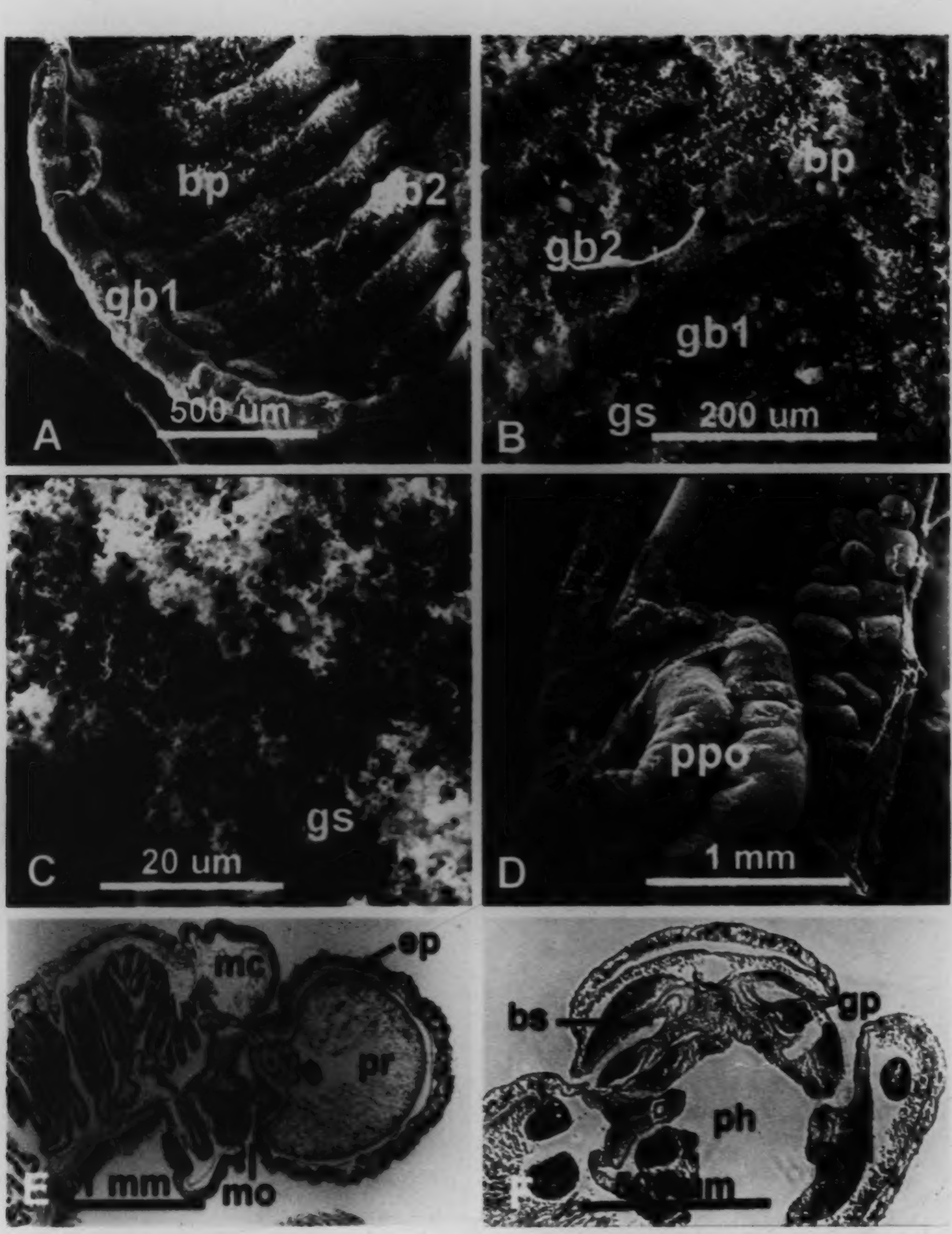
The enteropneust and cephalochordate pharynx share serially paired gill slits in the pharynx that are framed on either side with collagenous gill arches, and parted down the middle by a secondary gill (or tongue) bar. In ptychoderid enteropneusts (but not in *Harrimania*) synapticles, or collagenous bridges, join the primary bars with the secondary bars. The cephalochordate pharynx differs from that of the Enteropneusta in that it posses an endostyle on the ventral midline. Horst (1939) considered the ventral digestive gut of enteropneusts to be homologous to the chordate endostyle, but recent evidence indicates an endostyle-like organ in the *dorsal* pharynx of the enteropneust *Schizocardium brasiliense* (Ruppert et al. 1999). Further support for the dorso-ventral inversion hypothesis may be found in the direction of particle movement in the pharynx of H. planktophilus. The food is gathered on the gill bars and transported from dorsal to ventral (in chordates, they move from ventral to dorsal), and then transported posteriorly to the gut in a mucous food cord. The direction of food transport in *H. planktophilus* suggests that the enteropneusts are inverted dorso-ventrally with respect to the chordates and is supported by molecular evidence (Arendt and Nübler-Jung 1994). If the chordates are indeed inverted dorso-ventrally with respect to the enteropneusts, then the gill bars could not be considered homologous with those of amphioxus, because they would also be inverted dorso-ventrally and therefore contradict the conformational consistency required to assign homology. The coelomic diverticua in the gill and tongue bars is different in the two groups, suggesting that the gills of the common ancestor lacked primary gill arches and tongue bars. This idea is consistent with the absence of gill skeletal structures in adult pterobranchs, the adult enteropneust *Protoglossus*, urochordates and developmental stages of all other chordates. Clearly more comparative research is needed before we can be confident about gill arch homologies among the deuterostomes.

The enteropneust pharynx should not be considered less specialized than that of the chordate pharynx because it does not produce a mucous net. The pharynx of *H. planktphilus* is efficient at capturing a wide range of particle sizes, and although it does not have a mucous net, mucus is produced abundantly by the surface of the enteropneust body and surely plays a role in capture and transport of ingested material. The enteropneust pharynx may represent an intermediate stage in the evolution of the chordate pharynx, and the absence of the chordate mucus net may be due to the undue stress that it would receive during deposit feeding. *H. planktophilus* may not have a localized endostyle because the entire phayrnx lining produces mucous, effectively coating the interior of the pharynx in a manner that is analagous to a net. Alternatively, enteropneusts may simply have not evolved the ability to produce a mucus net. In any case, *H. planktophilus* seems to suspension feed perfectly well without one.

H. planktophilus are perhaps unique among the suspension feeding protochordates because they live entirely beneath the sediment. In aquaria, their burrows do not approach the sediment surface. Most investigations that quantify suspension feeding deal with epifaunal organisms (including sessile, tube dwelling and free living organisms) or infaunal forms that have part of their body or tubes in direct contact with the epibenthos (examples include the bivalve *Mya arenaria*, the polychaete *Chaetopterus*, the phoronid *Phoronis viridis*) (Wildish and Kristmanson 1997). *H. maculosa* appears to exploit nutrients from interstitial pore water, a world of gastrotrichs, nematodes, and the bacteria rich water that thrives on the boundary of the REDOX layer (Levinton 1995). *H. planktophilus* is a very active enteropneust, does not smell of bromophenols, and responds negatively to very low light levels. When it is removed the burrow its gut is full of sediment. Sediment most probably composes the mass of the gut's content, but food sequestered from pore water may assume the most important role nutritionally (Barrington 1940). Many deposit feeders display adaptations that improve their efficiency in sediment processing and food absorption (Lopez and Levinton 1987) and the filtration of nutrient rich pore water, such as observed in H. planktophilus, may be more common than formerly realized.

Clearly the evolution of slits in the pharynx is one of the most important elements in appreciating the evolution of chordates. Gill slits and pores were an invention of the common ancestor of chordates and hemichordates. Harrimania planktophilus is a facultative suspension feeder, and a detailed comparison between a wide range of hemichordates will undoubtedly reveal further evolutionary innovations of the branchial pharynx. Researchers will do well to study the feeding behavior of other enteropneusts including the swimming enteropneust Glandiceps hacksii (Spengel 1909), the deep sea rock-pendulum worm, Saxipendium coronatum, which lives atop rocky outcrops and extends its long proboscis into the water column (Woodwick and Sensenbaugh 1985), and Harrimania maculosa, which lives on the under side of rocks in Alaska (Ritter 1900). Much remains to be learned about the form, function and origin of the filtering pharynx of deuterostomes.

Figure VII-1. Scanning electron microscope images (A - D) and light micrographs of sectioned material of *Harrimania planktophilus*. A. The doral branchial pharynx of *H. planktophilus* showing primary and secondary gill bars. B. The gill bars and slits in the dorsal branchial pharynx. C. details of the cilia and mucuc on a gill bar. D. The post - pharyngeal organ concentrates food in passage from the pharynx to the gut. E. Light micrograph of longitudinal section through the proboscis, collar and anterior pharyngeal region of the trunk. F. Light micrograph of a saggital section through the pharynx. bp, branchial pharynx; bs, branchial sac; ep, epithelium; gb1, primary gill bar; gb2 secondary (or tongue) gill bar; gp, gill pore; gs, gill slit; mc, mesocoel; mo, mouth; ph, pharynx; ppo, post - pharyngeal organ; pr, proboscis.



bp
b2
gb1
500 um
A
bp
gb2
gb1
gs
200 um
B
gs
20 um
C
ppo
1 mm
D
mc
ep
pr
1 mm
mo
E
bs
gp
ph
F

Figure VII-2. The flow of food coloring into the mouth and out of the gill pores of *Harrimania planktophilus*. Scale bar is indicated in A, time in tenths of seconds is indicated in frames A - F. c, collar; fc, food colouring; p, proboscis; pip, pipette tip; t, trunk.

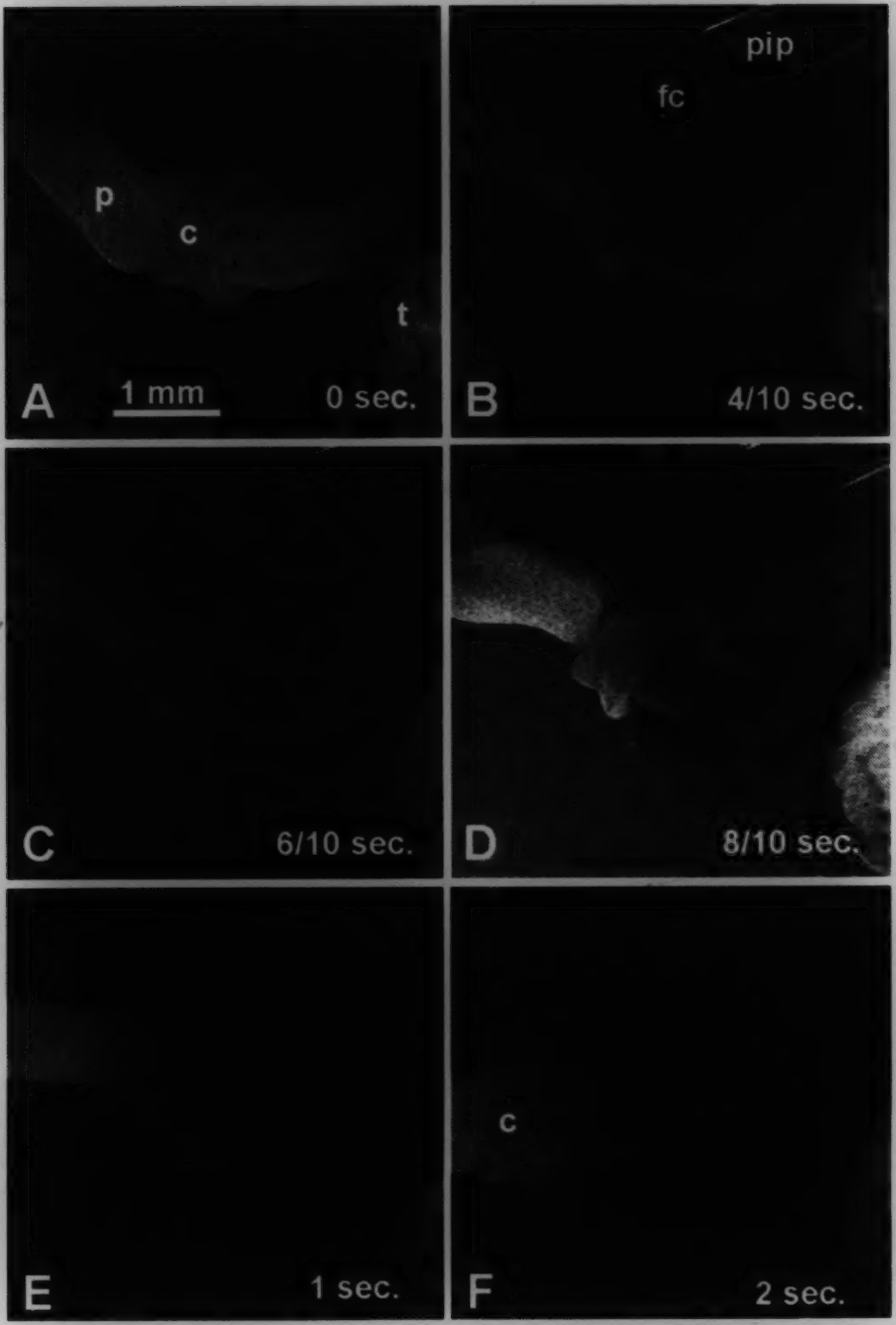
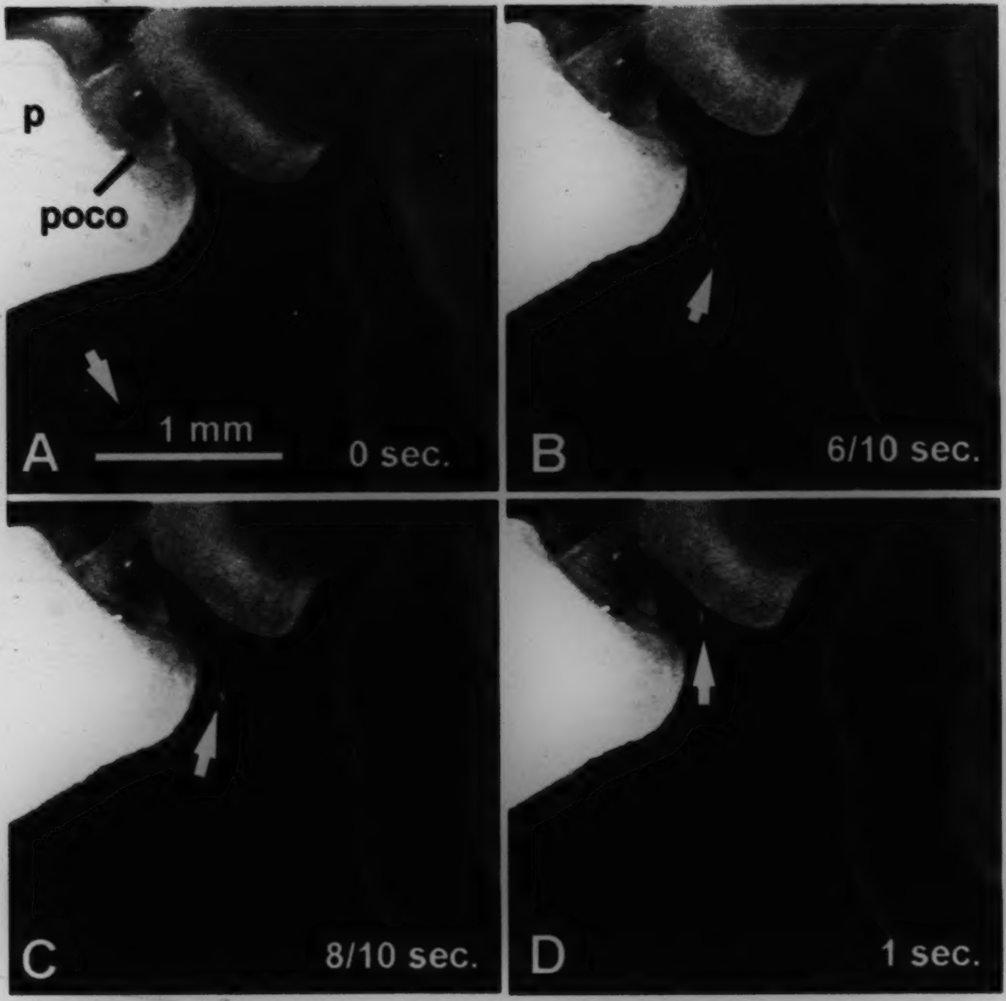

p
c
t
A
1 mm
0 sec.
pip
fc
B
4/10 sec.
C
6/10 sec.
D
8/10 sec.
E
1 sec.
c
F
2 sec.

Figure VII-3. Still photographs captured from video tape of particles moving towards the mouth of *Harrimania planktophilus*. Scale bar is indicated in A, time in tenths of seconds is indicated in frames A - D. c, collar; p, proboscis; poco, pre - oral ciliary organ; t, trunk. Arrows indicate the movement of a single particle in suspension as it is sucked towards the mouth.


p
poco
A
1 mm
0 sec.
B
6/10 sec.
C
8/10 sec.
D
1 sec.

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Chapter VIII

Conclusions

Cladists, Hemichordate Phylogeny, and Chordate Origins

The strengths of cladistic studies are not in the trees, but in the character matrix that ultimately provides the foundation for the trees. The character matrix presented in chapter II is an explicit collection of facts that are gathered from the literature and from my own studies on the hemichordates. The matrix provides a reference point from which others may readily observe areas of hemichordate research that have been completely ignored. For example, a quick look at the matrix will reveal that very little is known about the form of the coelomic cavities of the deep sea rock pendulum worm, *Saxipendium coronatum* and nothing about the development of many genera, including *Xenopleura, Stereobalanus* and *Protoglossus*. The data matrix is by nature an explicit set of assumptions about homology, and therefore can be modified to accommodate new information and differing opinions regarding symplesiomorphy assignment.

In this thesis I have used cladistic models to construct an evolutionary hypothesis of the deuterostomes, with particular emphasis on the little known phylum Hemichordata. A cladistic analysis of Hemichordata and higher deuterostomes was constructed using morphological characters (chapter II) and an analysis of the complete18S rDNA gene sequence (chapter III) under the assumptions of parsimony. Both studies strongly support hemichordate monophyly and place hemichordates as the sister taxon to the echinoderms. The difference between trees constructed from these independent data sets occurs within the hemichordate clade. Whereas the morphological data set suggests that the pterobranchs are the sister taxon to the enteropneusts (Figs. III-1A , B), the molecular topology suggests that the pterobranchs are sister taxon to the enteropneust family Harrimaniidae (Fig. III-2). Further discrepancy between data sets is indicated in the position of the enteropneust family Ptychoderidae. The morphological data set weakly supports the hypothesis that the Ptychoderidae occupy a basal position among the enteropneusts (Fig. II-2), differing from the 18S rDNA data set wherein the ptychoderids family (represented by *Balanoglossus* and *Ptychodera*) are sister group to the Pterobranchia plus the enteropneust family Harrimaniidae (Fig. III-2). The molecular tree implies that the Enteropneusta are basal among the hemichordates because it is more parsimonious to assume that the pterobranch morphology branched off of the harrimaniid clade than it is to assume the enteropneust morphology evolved twice from a pterobranch-like ancestor (once in the harrimaniids and once in the ptychoderids). The morphological tree suggests that the enteropneusts and pterobranchs are sister taxa and gives no reason to assume that either one represents the ancestral hemichordate morphology.

More work needs to be done before I will have confidence in either the morphological or the molecular phylogenetic trees presented in this thesis. In general, I think that the hemichordates are monophyletic and sister taxon to the echinoderms and an enteropneust - like morphology most likely provided the foundation for the evolution of the echinoderms, along one lineage, and the chordates along another. However, problems are incurred with cladistic analysis of morphological and molecular data sets. I have more confidence in the morphological reconstructions because the 18S rDNA gene has reconstructed suspicious trees among the protostome phyla (Abouheif and Meyer 1998). Furthermore, new evidence (28S rDNA) from a collaboration with J. Mallatt (Washington State University) supports the morphological phylogenetic hypothesis presented in chapter two.

Morphological characters hold clues to the intermediate stages between two character states, whereas molecular data only has to offer four discrete states for each nucleotide position. Morphological characters offer to us an indication of the evolution of the structure of a clade of organisms, whereas molecular data offers us the evolution of a gene within a clade and the jump between the evolution of a gene and the evolution of organisms is a leap of faith that few acknowledge. How natural selection operates on a population to form the evolution of a genus, family, class or phylum is a complex interaction of physiological and environmental variables of varying intensities across time. In general, gene trees should parallel species trees, but complications may arise due to selection and molecular convergence.

Ancestral character reconstruction using MacClade suggests that the common ancestor to the Deuterostomia had a morphology a tornaria - like and acorn worm - like morphology in its life history. A careful examination of the results from chapter II suggests that the common tornaria -like ancestor to the echinoderms (along one lineage) and the acorn worm - like ancestor to the chordates (along another) had gill pores, a notochord-like structure and a vestigial endostyle (the perioral band). This hypothetical ancestral reconstruction is corroborated by the presence of such an animal, *Tornaria hubbardi* (Ritter and Davis 1904), from the California coast.

Harrimania planktophilus **(n. sp.)**

Detailed studies of the form and function of enteropneusts yielded important insights into their biology and evolution. In chapter IV I describe a new enteropneust species (*Harrimania planktophilus*) from the family Harrimaniidae. Harrimaniids have no liver-sacs, no synapticles in the gill bars and no nerve-roots in the dorsal collar mesentery. All three species in the genus *Harrimania* (Ritter) have a short proboscis and lack genital wings. *H. planktophilus* and *H. maculosa* have collar mesenteries and a long neck skeleton that extends from the proboscis to the trunk. *H. planktophilus* can be distinguished from *H. maculosa* by a reduced branchial ridge and a reduced collar stomochord. *H. planktophilus* forms long sinuous burrows that are semi-permanent and shared. Females deposit a tubular egg mass in a burrow where the embryos develop (Fig. IV-4). Embryos were discovered at the late blastula stage. Invagination of the archenteron completely obliterates the blastocoel resulting a hemispherical shaped embryo (Fig. IV-3B). The embryo develops cilia and begins rotating around the embryonic fluid. *H. planktophilus* is significant to hemichordate biology because it has maintained the vestiges of an indirect life history, a diffuse larval swimming band, in it's ontogeny. Development of *H. planktophilus* was direct, and young juveniles hatched with a post - anal tail and assume an interstitial burrowing life habit. Adults had a robust body and possessed many large gill slits with well-developed tongue bars. This species may be most valuable as a model organism to study suspension-feeding in an enteropneust.

The Filter Feeding Pharynx Evolved Before the Divergence of Hemichordates and Chordates

One of the key events in chordate evolution has been the evolution of gill slits. Gill slits of chordates function in feeding (ascidians and cephalochordates), locomotion (salps and doliolids) (Bone 1998), and in gas exchange (vertebrates) (Radinsky 1987). The vertebrate pharynx provided a foundation from which evolved the vertebrate jaw, hyoid arches and thyroid gland (Radinsky 1997). A widely under-appreciated fact about the chordate pharynx is that it evolved in the common ancestor to the chordates and the hemichordates. It is generally assumed the enteropneust pharynx is involved in gas exchange (Ruppert and Barnes 1994), but physiological studies are completely wanting. The enteropneust pharynx is thought to provide an exit for excess water that is ingested from a deposit feeding diet (Knight-Jones 1953), yet many other taxa, including echiurans, holothuroids and polychaete worms deposit feed in an analogous way to enteropneusts, without the help of pharyngeal pores.

In this thesis I show that *Harrimania planktophilus* exhibits a feeding behavior similar to that described in protochordates, and strongly suggest that the existence of the filter feeding pharynx preceded the evolution of the chordates. *H. planktophilus*, the 'plankton loving' enteropneust that is described in chapter IV, uses cilia lining the pharynx to pump food laden water at velocities of 0.5 - 2.0 mm / second. Particles as small as 0.2 µm and as large as 28.4 µm are effectively filtered and transported to the gut with a dense covering of cilia on the pharyngeal bars. *H. planktophilus* has evolved the ability to effectively filter particles from suspension without an endostyle or mucus net. This study indicates that the suspension feeding pharynx is not an innovation of the chordates, but instead intermediate stages that lead to the chordate pharynx evolved in the common ancestor to the chordates and hemichordates.

The Nervous System of *Saccoglossus* sp.

A species of *Saccoglossus* from Barkley Sound, British Columbia, was observed in the field and found to exhibit a startle withdrawal response. Optical and electron microscopy of the nerve cords failed to reveal giant axons. The dorsal collar cord and ventral trunk cord consist of small axons with a mean diameter of 0.4 µm. The majority of the axons run longitudinally and there is no indication of a specialized integrative center. Electrical recordings from the nerve cords show events interpreted as compound action potentials. The potentials are through-conducted from proboscis to trunk. Such propagated events probably mediate startle withdrawal. Conduction velocities did not exceed 40 cm • s^{-1} in any part of the nervous system. The results of this study suggest that the dorsal nerve cord of *Saccoglossus* cannot be compared to the central nervous system of chordates, but instead, the enteropneust nervous system is comparable to the ectoneural system of echinoderms.

Mulberry cells are epidermal gland cells bearing a long basal process resembling a neurite and are tentatively regarded as neurosecretory cells. They occur scattered through the ectoderm of the proboscis, collar and anterior trunk regions of the acorn worms *Saccoglossus*, usually in association with concentrations of nervous tissue. They contain secretion granules that appear from electron micrographs to be released to the exterior. The granules are immunoreactive with antisera raised against mammalian and salmon gonadotropin-releasing hormone (GnRH). Similar results were obtained with another enteropneust, *Ptychodera bahamensis*, using antisera raised against tunicate-1 and mammalian GnRH. Mulberry cells were not found in either *Cephalodiscus* or *Rhabdopleura* (Hemichordata: Pterobranchia).

Extracts of tissues from 4200 *Saccoglossus* contain an area of immunoreactive GnRH that is detected by an antiserum raised against lamprey GnRH when characterized by high performance liquid chromatography and radioimmunoassay.

This is the first report of the occurrence of GnRH in hemichordates, probably the most primitive group clearly belonging to the chordate lineage. The physiological function

of GnRH in enteropneusts is unknown, but an exocrine function appears more likely than an endocrine or neurotransmitter role.

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Appendix I

Brominated Compounds Secreted by *Saccoglossus* sp. Deter Fish Predation, Inhibit Aerobic Bacterial Growth, and Filter Colonization of Sympatric Sedimentary Macrofauna*

Abstract Almost all species of enteropneusts investigated contain holoaromatic compounds and secrete them into their environment. Bromophenols are particularly common and their apparent ubiquitous distribution in the enteropneusts is presumably because they impart some benefit to the worms that outweighs the cost of sequestering and /or producing them. We conducted three tests of the functional significance of bromophenols in an udescribed species of *Saccoglossus*; (i) palatability to predators, (ii) effect on aerobic bacterial growth, and (iii) effect on recruitment and colonization in the field. The crab, *Cancer magister* was not deterred by the *Saccoglossus* sp. tissue, but shiner perch (*Cymatogaster aggregata*) and prickly sculpin (*Cottus aspen*) rejected it. Bromophenols inhibited growth of aerobic bacteria that were cultured from the marine sedimentary environment. In the field colonization study the number of species did not differ significantly between experimental treatments containing *Sacoglossus* sp., treatments containing diffusable halogenated compounds (but no *Saccoglossus* sp.), and control treatments. Animal abundance, on the other hand, decreased by about one half between control treatments and brominated sediments, and was lowest in treatments containing *Saccoglossus* (and therefore exposed both to halogenated compounds and to the mechanical disturbance of the worm). The decline in overall animal abumdance, however, was due almost entirely to a precipitous drop in spionid numbers. The negative response of fish predators, anaerobic bacterial growth, and recruitment of spionid polychaetes to brominated compounds suggests that brominated compounds have wide ranging effects on saccoglossid ecology.

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Introduction

Many animals secrete highly toxic halogenated compounds (all brominated), including corals, members from at least eight polychaete families, phoronid worms, and a prosobranch mollusc (Woodin et al. 1987, Fielman et al. 1999). Surprisingly, almost all species of enteropneusts investigated have large quantities of the toxins. The mucus linings that stabilize the well developed burrows of enteropneusts smell of these compounds (Brambell and Cole 1939, Knight-Jones 1953, Ashworth and Cormier 1967, King 1986). It is not clear if the worms are metabolizing the halogenated compounds or sequestering them from their environment. Although the structure of these compounds varies considerably among species (Higa 1985, Sharief 1997, Giray and King 1987a) bromophenols seem to be particularly important to their ecology.

Considering the toxicity of these compounds (the related chlorinated phenols are well known for their potency as disinfectants) it is not understood how these animals survive their own secretions. Perhaps the most intuitive function of the toxins is to reduce or completely inhibit microbial and microfaunal degradation of the burrow-wall mucous lining or to alter local biogeochemistry (King 1986). In addition, oxidation of ferrous iron would result in the deposition of iron oxyhydroxides that provide a better barrier to sulfide influx into the burrow (Giray and King 1997a). As expected, the burrow wall of *Saccoglossus kowalevskii* is lined with a 2 mm thick deposit of iron oxyhydroxides (King 1986). King (1986) also pointed out that decreased microbial metabolism would increase the longevity of mucus linings. Presumably a decreased requirement for replacing mucus could result in greater allocation of energy for growth and reproduction.

Jensen et al. (1992) were struck by the manner in which somewhat similar burrows systems produced by an echiuran and an enteropneust in the same sediment had contrasting microbial and meiofaunal colonization. The echiuran burrow walls contained a diverse community of nematodes, foraminifera and bacteria, whereas the toxic walls of the enteropneust were devoid of symbionts.

Giray and King (1997b) showed that bromophenols in the enteropneusts *Saccoglossus bromophenolosus* and *Protoglossus graveolens* did not deter predation by a hermit crab and two polychaetes from the family Nereidae. The resilience of nereid polychaetes to halogenated aeromatics is not apparent in the juveniles of *Nereis vexillosa*, for they will avoid recruiting to sediments that are contaminated with brominated metabolites from the polychaete *Thelepus crispus* (Woodin et al. 1993).

The objective of this study was to determine if the presence of bromophenols in the tissue of *Saccoglossus* sp. deter predation by predatory fish and crabs commonly found in microhabitats containing *Saccoglossus* sp., and furthermore to determine if the presence of bromophenol in and around *Saccoglossus* sp. inhibits colonization of bacteria and macrofaunal species commonly found in association with *Saccoglossus* sp.

Materials and Methods

Individuals of an undescribed species of saccoglossid, *Saccoglossus* sp. were collected subtidally in the Ross Islets (49^{o}52' N, 125^{o}10' W), Barkley Sound during the summer months of 1998 and 1999 using SCUBA and transported to aquaria where they were cleaned of debris and allowed to empty their guts of sediment. Three experiments were designed: (i) to observe the effect of bromophenols isolated from *Saccoglossus* sp. on marine aerobic bacterial growth, (ii) to test the palatability of *Saccoglossus* to various predators, and (iii) to assess the impact of bromophenols on the colonization of sediment in the field by sympatric species.

Microbial experiment

Isolation of Marine Bacteria

Seawater samples were collected in sterilized glass bottles off of the Bamfield Marine Station docks, from which 200 µm sub-samples were plated on Trypticase Soya Agar (TSA) media and incubated at 37 ˚C for 24 hrs. The resulting colonies were selected and repeatedly streak -plated until one colonial morphotype (Table 1) was isolated.

Extraction

The enteropneust worms were weighed and homogenized with a mortar and pestle in 2 ml of 100% methanol. The homogenate was vortexed for 1 minute and stored in the refrigerator for 24 hrs. After 24 hrs, the homogenate was centrifuged for 15 minutes at 3000 rpm, and the resulting supernatant was transferred to sterilized test tubes.

Preparation of bromophenolated paper disks for bacterial growth assays

Control bromophenol concentrations were based on those found in the sister species of *Saccoglossus* sp., *S. kowalewskii* (9.9 µmol per gram fresh wieght) (King 1986). Using 10 µl of bromophenolated supernatant per 6 mm diameter paper disk, bromophenol concentration was determined to be 0.9447 µl per 10 µl of methanol. Based on one-hundred 6 mm disk volume (10 µl per disk X 100 disks = 1000 µl), varying stock bromophenol concentrations were prepared ranging from 1:16 to 8:1 times known concentration (Table 2). A 10 µl aliquot was added to a sterilized 6 mm paper filter disk and allowed to dry at room temperature.

The supernatant from the worm extract was prepared so that it had the same concentration (1:1) as determined for *Saccoglossus kowalewskii* (King 1986). Each 6 mm disk was wetted with a 10 µl aliquot of assumed to have 0.9447 µl of bromophenol. Using the weight of the worms determined in the worm extraction procedure, the equivalent weight was determined with the appropriate number of 6 mm disks. The total number of disks needed to match the worm weight was then multiplied by the 10 µl per disk aliquot to

give the final volume for the worm extract. A 10 µl aliquot was added to a sterilized 6 mm paper filter disk and allowed to dry at room temperature.
ex: 0.1 g worm = 40 disks and 40 disks x 10 ul = 400 ul.

Bioassays

The diffusion plate method (Zubay 1988) was used to determine the effectiveness of the bromophenols at inhibiting growth of the isolated marine bacterial strain. The bacteria were diluted 8 - fold in 2 ml of 0.5 µm millipore filtered and autoclaved seawater. One hundred µl of the final dilution was plated on TSA media made with 0.5 µm millipore filtered and autoclaved seawater. Millipore paper disks were gathered with a standard hole puncher and wetted with control bromophenol concentrations. The bromophenolated disks and worm extract were allowed to dry and then placed onto individual plates. The plates were incubated at 37 °C for 24 hours.

After incubation, the diameter of the zones of bacterial inhibition were measured. The zone of inhibition was characterized by a circle of agar that was free of bacterial growth surrounding the paper disks and was measured from the center of the disk to the closest zone of bacterial growth. Each known bromophenol concentration was run ten times, while each worm extract was run only once but repeated 10 times with 10 different worms.

Palatability experiment

To determine if *Saccoglossus* sp. was palatable to shiner perch (*Cymatogaster aggregata*), prickly sculpin (*Cottus aspen*), and a brachyuran crab (*Cancer magister*), we first trained the predators to eat small pieces of mussel (*Mytilus trossulus*) and then randomly interspersed their mussel diet with small pieces of acorn worm. Ten buckets were arranged in a sea water table, each with aeration and clean water flow. Each bucket contained one predator that was fed a small piece of mussel every second day. If the predator did not eat the mussel immediately upon feeding the food was removed. Once the food was eaten immediately for three consecutive feedings the predator was then fed a piece of worm. Again, if the worm was not taken into the mouth then it was removed from the bucket. A predation result was only recorded when the worm was taken into the mouth of the predator. The feeding event was either recorded as positive (the worm was eaten by the predator) or negative (the worm was ingested and then rejected by the predator). Once each individual was tested with the worm, the worm was removed (if rejected) and a piece of mussel was dropped into the tank to ensure that the predators were still continuing to ingest prey and not just rejecting everything that day. Once a rejection response was observed, the predator was returned to it's natural environment and no longer used in feeding trials.

Colonization experiment

Twenty pairs of plastic margarine containers (10 in the summer of 1998, and 10 in the summer of 1999) each having a nitex mesh (1mm) partition that physically separated one half of the container from the other were filled with autoclaved sediment that was collected from a site where *Saccoglossus* sp. occur. Two containers (each divided into two equally sized chambers) were fastened to a plastic board with epoxy and a screw. In one hemicylinder a pair of *Saccoglossus* sp. were introduced and allowed to establish a burrow while in aquaria. The hemicylinder adjacent to *Saccoglossus* sp. was left void of worms so that the effect of bromophenols on colonization could be determined in the absence of the bioturbation and feeding activity of the worms. The second container (having 2 hemicylinders) contained sediment without worms, and therefore no bromophenols, and functioned as a replicated control to test the magnitude of random variation in colonization rates between hemicontainers. Ten plastic boards, each with a pair of containers were then transported to the field where they were buried so that the level of sediment in the container equaled that of the natural environment. In the summer of 1998 five treatments were buried in a boulder field and 5 in a open sedimentary environment (in the absence of rocky substrate) at 10 meters depth. In the summer of 1999 all 10 treatments were buried in the open sedimentary environment. After two months in the field (6-14-98 to 8-20-98 and 6-20-99 to 8-24-99) the treatments were collected and brought back to the lab for sorting. Animals that had colonized the containers were removed, identified to species or nearest taxonomic level and recorded by treatment (*Saccoglossus* present, *Saccoglossus* adjacent, control A, control B).

The diversity of colonists was calculated with the Shannon index of diversity, H' (Zar 1999). To test the null hypothesis whether the diversities of the two sampled populations were equal we calculated a one - tailed Hutchinson's t - test (Zar 1999) where:

$$t = \frac{H'_1 - H'_2}{\sqrt{(S^2_{H1} + S^2_{H2})}}$$

, and $S^2_{H'}$ is the variance for each indices.

The variance in H' was computed as the variance among the replicate hemicontainers for each treatment.

Results

Microbial experiment

2,4 - Dibromophenol (Sigma) inhibited aerobic bacterial growth most effectively in areas surrounding paper disks of highest concentration (8: 1) creating a zone of inhibition with a mean of 53.26 mm (SE = 0.415). A linear decrease in the zone of inhibition occurred as the 2,4 - dibromophenol was diluted to lowest experimental treatment (Figure 1). Paper disks that were treated with mucus from *Saccoglossus* inhibited bacterial growth in an area with a radius of 13.84 mm (SE = 0.834), suggesting that the concentration in worm tissue was close to 0.62 µmol / g fresh weight.

Palatability experiment

To test the palatability of *Saccoglossus* sp. tissue we chose two fish species and a crab that are commonly found feeding in *Saccoglossus* sp. habitat. Ten shiner perch (*Cymatogaster aggregata*) rejected the worm. Ten prickly sculpin (*Cottus aspen*) rejected the worm whereas all 16 brachyuran crabs (*Cancer magister*) ate the worm.

Colonization experiment

To determine if infaunal diversity differed between chambers containing *Saccoglossus* sp., adjacent to *Saccoglossus* sp. (and therefore containing diffusible allelochemicals from *Saccoglossus* sp. but not affected by the mechanical activity of *Saccoglossus* sp.), and experimental controls we tested for diffences in the Shannon index of diversity (Zar 1999). Control A was used for all comparisons because it was the more conservative of the two controls.

Control A was not significantly different from the treatment adjacent to *Saccoglossus* sp. ($t_{0.05(1),280} = 0.545$, $p > 0.25$). The adjacent treatment was significantly different than the treatment with *Saccoglossus* sp. present ($t_{0.05(1),256} = 3.26$, $p < 0.0005$). Control A was significantly different from the treatment with *Saccoglossus* sp. present ($t_{0.05(1),285} = 4.30$, $p < 0.0005$).

A significant difference in the total number of animals was observed between control A and adjacent treatment, between control A and *Saccoglossus* sp. containing treatment, and between adjacent treatment and *Saccoglossus* sp. containing treatment (Table 3). The significant difference in total numbers of animals observed between treatments was primarily due to colonization by the spionid polychaete *Prionospio steenstrupi* which increased in numbers from *Saccoglossus* sp. inhabited containers (59) and *Saccoglossus* sp. adjacent (88) with the highest numbers in the controls (181 and 184). The Maldanid polychaete *Praxillella praetermissa* also increased in numbers from the container containing *Saccoglossus* sp. (6), to adjacent *Saccoglossus* sp. (12) with the highest numbers in the controls (14 and 14). The Ophelid polychaete *Armania brevis* also had the highest numbers in the controls, although the trend was somewhat weaker. Only a single Goniadid polychaete (*Glycinde* sp.) was found in a control and another in a container adjacent *Saccoglossus* sp., whereas fifteen of the animals were found in containers containing *Saccoglossus* sp.. The phyllodocid *Phylodoce groenlandica* was also most abundant in the *Saccoglossus* sp. treatment (12 animals) whereas the controls had 5 and 6, but only a single animals was collected from the adjacent treatment. However, except for *Prionospio*, none of the differences in abundance of individual species among treatments was significant statistically. Also, no significant difference was observed in the number of species among the four experimental treatments (Table 3).

Discussion

The presence of bromophenols in the sediment may have direct effects or indirect effects on the sympatric community. For example, *Saccoglossus* worms produce allelopathic effects through the chemical agent bromophenol, secreted from cells of the body and exuded with mucus to the lining of the burrow walls and diffusing into the surrounding sediments (King 1996). Other effects may be indirect, for example reducing aerobic activity can decrease biological oxygen uptake, resulting in a zone of chemical sulfide and ferrous iron oxidation (King 1986). This in turn would increase the influx of toxic hydrogen sulfide (H_2S) from reduced sediments adjacent to the burrow of *Saccoglossus* sp., but not into the burrow because of an iron oxyhydroxide lining (King 1986), and slow the invasion and replacement of this sedimentary community by other species.

In this study we measured the direct effects of 2, 4 - dibromophenols on bacterial growth and compared it to the effect of the mucus of *Saccoglossus* sp. on bacterial growth. Although many chemicals may be secreted with the mucopolysaccarides of *Saccoglossus* sp. (Cameron et al. 1999), bromophenols seem to be the most prominent allelochemical found in relatively high concentrations in all enteropneusts investigated (Ashworth and Cormier 1967, Higa et al. 1980, Corgiat et al. 1993). 2, 4 dibromophenol was found to inhibit larger areas of aerobic bacterial growth in high concentrations and smaller areas in diluted concentrations (Table 2, Figure 1), corroborating the findings of King (1986). Mucus secreted from *Saccoglossus* sp. was found to inhibit bacterial growth with similar intensity of 2, 4 - dibromophenols at a concentration between 11.81 and 5.90 µl g -1, suggesting that the concentrations of bromophenols in the mucus of *Saccoglossus* sp. are similar to those concentrations quantitatively observed in *Saccoglossus kowalevskii* (King 1986) and *Balanoglossus biminiensis* (Ashworth and Cormier 1967). Many soils contain substances, presumably accumulated antibiotics, that exert a moderate, general background inhibition of bacteria and fungi (Christophersen 1983). Although only speculation, the apparent antibiotic quality of seawater (ZoBell 1946) may be due in part to the metabolic products of sedimentary organisms.

Little is known about predation on enteropneusts in situ. The snail *Terebra dislocata* preys on the posterior trunk of *Balanoglossus aurantiacus* as it projects from the borrow to defecate (Ruppert and Fox 1988) and species of conus snails have been reported to feed on enteropneusts (Kohn 1983). Giray and King (1997b) documented the ineffectiveness of dibromophenols at deterring predation of *Saccoglossus bromophenolosus* and *Protoglossus graveolens* by the anomuran crab *Pagurus* *longicarpus*, and the polychaetes *Glycera dibranchiata*, *Nereis virens* and *Nephtys incisa*. In this study we also found that small pieces of *Saccoglossus* sp. were readily ingested by the brachyuran crab *Cancer magister*, but they were unequivocally rejected by shiner perch (*Cymatogaster aggregata*) and the prickly sculpin (*Cottus aspen*). Chemical defenses are frequently employed through sprays, bites, stings and volatile secretions. Like many animals whose defense is directed towards predators with the ability to learn, *Saccoglossus* sp. is aposematically coloured (especially in the vulnerable proboscis) presumably to help the fish predator avoid future mistakes.

In the darkness of the sediment, colours and differences in structure among organisms are much less prominent than they are above ground. The space to which infaunal organisms relate is defined largely by chemical gradients and it is clearly necessary to approach through biochemical adaptation both the niche differentiation of infaunal organisms and their integration into the sedimentary community as a functional system. In communities in which a number of infaunal species are mixed together, these may form a mosaic of differing chemical effects on the sediment which may contribute (along with sediment characteristics, food availability, predation, and so forth) to the patterning and species diversity of the epifauna (Woodin et al. 1993). Brominated metabolites have been implicated in the reduction in the numbers of meiofauna organisms in the burrow wall linings of the deep sea enteropneust *Stereobalanus canadensis* (Jensen et al. 1992).

In this study we were interested in the effects of allelochemicals, primarily bromophenols, on colonization in sediments with *Saccoglossus*, adjacent to *Saccoglossus* sp. and without *Saccoglossus* sp.. The experimental chambers adjacent to *Saccoglossus* sp., but containing no *Saccoglossus* sp., were integral to the experiment because they allowed us to see the effects of diffusable bromophenols in the absence of the mechanical activity of the worms (feeding, burrowing, defecation etc...). Chambers containing diffusable secreted products from *Saccoglossus* sp. (but no *Saccoglossus* sp.) had dramatically fewer total numbers of animals colonizing them compared to control chambers (Table 3), although no significant difference in diversity was found. The differences in total number of animals observed between treatments was almost entirely due to colonization by the spionid polychaete *Prionospio steenstrupi*, the dominant member of this infaunal community (Table 4.). The phyllodocid polychaete *Phylodoce groenlandica* was observed in the lowest numbers in the chambers adjacent to *Saccoglossus* sp. and the highest numbers in chambers containing *Saccoglossus* sp., suggesting that the selective advantage gained from co-occurring with *Saccoglossus* sp. outweighs the disadvantage of bromophenol toxicity. Bromophenols may have no serious disadvantage for errant or successional species, since these are vagabond populations that dominate a community for a short period in a given location. Similarly, the goniadid polychaete, *Glycinde* sp. was found almost exclusively in treatments containing *Saccoglossus* sp., suggesting that the mechanical activity of the worm, or the succession of species associated with the worm, provided some favorable condition.

Bromophenols provide enteropneusts with a broad spectrum effects against microbial, meiofaunal and macrofaunal organisms living in the sediment. They deter predation by predatory fish, and settlement by nereid polychaetes (Woodin et al.1993), the same family of polychaete known to feed on *Saccoglossus bromophenolosus* (Giray and King 1997b). It is not known what the effects of brominated metabolites may have on viruses and fungi, parasitism, intraspecific communication, selection of habitat, and food and dispersal. The secretion of bromophenols by *Saccoglossus* illustrates the vital role of such chemicals in many kinds of interspecific interactions, including predation, competition, defense and organization of communities. The natural environment is a maze of chemical stimuli that may effect the survival of many species. The role of form and colour in adaptation has long been known (LaBarbera 1984, Reimchen 1989) but more recent research with advanced chemical techniques has elucidated some of the variety of chemical adaptations that are at least equal significance in the interrelations of species in natural communities.

Table AI-1. Characteristics of the aerobic marine bacterium isolated to test for inhibitory growth effects of 2, 4 - dibromophenol and mucus extracted from *Saccoglossus* sp.

Colonial Morphologies	
Size	6mm @ 24 hrs.
Shape:	Circular
Pigmentation:	White
Elevation:	Raised
Surface:	Smooth
Optical Characteristic:	Opaque
Emulsifiability:	Easy
Odor:	Absent
Cellular Morphologies	
Shape:	Staphylocococcus
Gram Stain:	Positive

Table AI-2. Concentration of 2, 4 - dibromophenol added to paper disks to test growth inhibition of aerobic bacteria plated on agar media.

Bromophenol Concentation	Volume of Methanol (µl)	Volume of Bromophenol (µl)
1:1	905.53	94.47
2:1	811.06	188.94
4:1	622.12	377.88
8:1	244.24	755.76
1:4	976.38	23.62
1:8	988.19	11.81
1:16	994.10	5.90

Table AI-3. Shannon index of diversity calculated from the pooled data from summers 1998 and 1999. Total number of individuals and total numbers of species retrieved from 13 treatments (five from the summer of 1998, and eight from the summer of 1999). One treatment was recovered from the rocky site, and 12 from the soft bottom sites.

Treatment	Diversity index	# of individuals		# of species
	mean	total	excluding spionids	
control A	0.596	259	51	21
control B	0.532	245	46	20
adjacent to *Saccoglossus*	0.637	132	29	19
Saccoglossus present	0.904	124	54	22

Table AI-4. Distribution of taxa that colonized control sedimentary containers, hemicontainers adjacent to *Saccoglossus* sp. (and therefore permeated with diffusable halogenated compounds) and containers containing *Saccoglossus* sp. (and therefore susceptible to diffusable halogenated compounds and to the mechanical activity of the worms).

Taxon	Control A	Control B	Adjacent to *Saccoglossus*	*Saccoglossus* Present	Total
OPHIUROIDEA					
Amphiodia occidentalis	2		1	1	4
CRUSTACEA					
Cancer branneri	2				2
Pagurus sp.	1		2		3
Amphipoda	3		2	2	7
Dendrobranchiata	5	6	2	3	16
MOLLUSCA					
Calliostoma sp.	1	3		1	5
Clinocardium nuttalli	4	1	1	2	8
Nassarius sp.				1	1
Tegula brunnea		1	1		2
Tresus capax	3	3	4	3	13
Bilvalvia		3	1		4
Scaphopoda	1				1
Gastropoda	1				1
NEMERTEA					
Cerebratulus sp.			1		1
Paranemertes peregrina		1		1	2
SIPUNCULA					
Phascolosoma agassizii	1				1
POLYCHAETA (family)					
Glycera sp. (Glyceridae)	3	3	2	3	11
Glycinde sp. (Goniadidae)		1	1	12	14
Lumbrineris (Lumbrineridae)				1	1
Nephtys ferruginea (Nephtyidae)		1		1	2
Armania brevis (Ophelidae)	7	13	5	5	30

Phyllodoce groenlandica (Phyllodocidae)	6	5	1	12	24
Syllis sp. (Syllidae)		1	1	1	3
Terebellidae	5	1	2	3	11
Praxillella praetermissa (Maldanidae)	14	14	12	6	46
Oweniidae	11	3	5	3	22
Pectinariidae	3	2		1	6
Polynoidae	2	1		3	6
Prionospio steenstrupi (Spionidae)	184	181	88	59	512
TOTAL	259	244	132	124	759

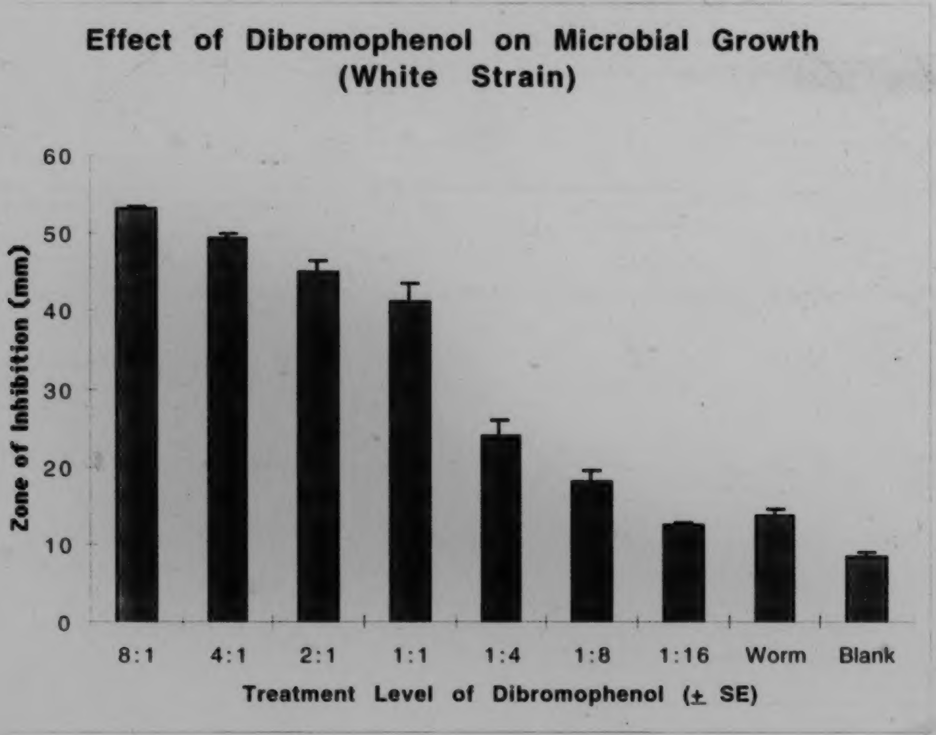

Figure AI-1. The mean area of inhibition of aerobic bacterial growth by decreasing concentrations of 2, 4 - dibromophenol, of mucus collected from *Saccoglossus* sp., and of paper disks without 2, 4 - dibromophenol or *Saccoglossus* mucus. The 1:1 concentration of dibromophenol is 9.9 μmol / g fresh weight. N = 10 for all bars.

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